

## Contribution à l'épidémiologie de la tuberculose bovine dans le nord de l'Equateur



## Contribution to the epidemiology of bovine tuberculosis in northern Ecuador



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# Contribution to the epidemiology of bovine tuberculosis in northern Ecuador

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This thesis is dedicated to my son Emilio Alejandro and  
my daughter Arianna Daniela, who are my inspiration

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# TABLE OF CONTENTS

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ACKNOWLEDGEMENTS .....	iv
TABLE OF CONTENTS.....	v
SUMMARY .....	vii
RÉSUMÉ .....	x
RESUMEN .....	xiv
LIST OF ABBREVIATIONS .....	xviii
CHAPTER 1 – INTRODUCTION.....	1
1.1 History of bovine tuberculosis .....	2
1.2 Taxonomy and characteristics of the <i>Mycobacterium bovis</i> .....	4
1.2.1 Characteristics of <i>M. bovis</i> .....	6
1.2.2 <i>M. bovis</i> genome .....	7
1.3 Pathogenesis .....	9
1.4 Epidemiology .....	10
1.4.1 Prevalence of BTB in cattle .....	10
1.4.2 Transmission of BTB among cattle .....	14
1.4.3 Prevalence and transmission of BTB among non-bovine species .....	17
1.4.4 Prevalence and transmission of BTB among humans .....	20
1.5 Diagnosis .....	22
1.5.1 Ante-mortem ( <i>in vivo</i> ) diagnosis .....	22
1.5.2 Post-mortem inspection .....	29
1.5.3 Laboratory diagnostic tools .....	33
1.6 Control of BTB .....	46
1.7 Eradication .....	49
CHAPTER 2 – OBJECTIVES.....	51
CHAPTER 3 – RESULTS.....	53

3.1 Preliminary observations on <i>Mycobacterium</i> spp. in dairy cattle in Ecuador .....	54
3.2 Comparative intradermal tuberculin test in dairy cattle in the north of Ecuador and risk factors associated with bovine tuberculosis .....	61
3.3 Post-mortem examination and laboratory based analysis for the diagnosis of bovine tuberculosis among dairy cattle in Ecuador .....	69
3.4 Single <i>Mycobacterium bovis</i> strain prevailing among dairy cattle in northern Ecuador .....	78
3.5 Risk analysis for infection of <i>Mycobacterium</i> spp. in a human population in Ecuador .....	85
3.6 Review article: Situation of bovine tuberculosis in Ecuador .....	102
CHAPTER 4 – DISCUSSION AND PERSPECTIVES .....	124
CHAPTER 5 – SYNOPSIS .....	135
CHAPTER 6 – REFERENCES .....	142

## SUMMARY

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In Ecuador, as in other Latin American countries, bovine tuberculosis (BTB) is a major concern in the dairy cattle industry, with economic and public health importance. The increase of peri-urban dairy production associated with the absence of BTB control program in Ecuador are major risk factors in the context of tuberculosis. Furthermore, official data related to the disease are scarce and there is no national veterinary reference laboratory for mycobacteria in Ecuador. The present work was undertaken in this context. The main objectives were to provide data on the epidemiologic situation of BTB among dairy cattle in northern Ecuador, and to compare the performances of the standard diagnostic tools used to identify *M. bovis*.

In a first step, a skin test survey was performed in 2003 in the Mejia canton, the major dairy cattle production region of Ecuador. Randomly selected 1012 cattle from 59 dairy herds, classified according to herd size, were tested for tuberculosis by the single intradermal tuberculin test (SITT) and the comparative intradermal tuberculin test (CITT). Results demonstrated 7.95 % of tuberculin skin-reactors in large herds *versus* 4.24 % in medium and 0.30 % in small herds. These first results underlined the importance of BTB in dairy cattle in the Mejia canton with herd size as a probable risk factor.

A more detailed field investigation was performed in 2007 and 2008 in the same region. A total of 2022 cattle from 20 dairy herds were studied. In 2007, each animal was tested using CITT and a follow-up test was performed in the same herds in 2008. The true annual

incidence was 1.70 %. Analysis of CITT results showed significant differences between medium and large herds with a BTB prevalence of 0.27 % and 0.57 % in medium-sized herds compared with 8.63 % and 8.43 % in large herds. Herd size was identified as a significant risk factor, confirming the results of the first study. The number of skin test-positive cases also increased significantly with age, contacts with other animal species and introduction of new cattle into the herd.

A third study was carried out in 1390 slaughtered cattle over a two years period. Classical diagnostic tools were compared to detect infected cattle. A total of 33 animals with gross suspected lesions were detected representing 2.3 % and 2.4 % of animals inspected in 2007 and 2008, respectively. From these suspected cases, acid-fast bacilli (direct microscopy) were identified in 33% (11/33) and suggestive microscopic lesions (histopathology) in 27.3 % (9/33) of the cases. Cultures and PCR yielded 36.4 % (12/33) and 27.3 % (9/33) of positives, respectively. Overall, the combined use of microscopy, histopathology, PCR and culture identified infected cattle (at least one positive test) in 51.5 % (17/33) of the suspected animals. Compared to culture, other laboratory procedures displayed a sensitivity of 56.5 % (PCR) and 43.5 % (microscopy and histopathology) and a specificity of 94.4 % (PCR and microscopy) and 97.2 % (histopathology). These results underline that reliable post-*mortem* laboratory testing either requires the combination of several tests or necessitates the development of improved tools with better performance characteristics. It should be noted that lesions were mainly observed in thoracic lymph nodes suggesting that the respiratory tract is the main route of transmission in dairy cattle inspected at slaughterhouse.

In a subsequent step, spoligotyping was used to compare *M. bovis* strains. All strains (n = 23) isolated from slaughtered cattle from Mejia canton presented the same spoligotype (SB0980).

Such homogeneity could be related to the relatively small study area. A more extensive molecular study, at a national level, could be helpful to assess the strain diversity of *M. bovis* in cattle populations in Ecuador.

In countries where BTB controls are lacking, *M. bovis* is also of public health concern. The last study was undertaken to evaluate the prevalence of *Mycobacterium* spp. in risk populations by tuberculin skin test (TST). The study was conducted on 157 people (farm and slaughterhouse workers) and TST revealed a high prevalence of reactors (29 %). The main risk factors associated with positive human reactors were consumption of raw milk or handmade cheese. Campaign informations are needed to make rural populations aware of the zoonotic risk related to *M. bovis*.

In conclusion, data presented in this thesis confirm the importance of BTB in dairy cattle in northern Ecuador and highlight the need for a national BTB control program. Ideally, this program should include (1) a reference laboratory for BTB and the training of qualified veterinarians; (2) mandatory skin testing and culling of reactors with official compensations; (3) detailed post-mortem inspection with laboratory-based diagnosis associated with a trace-back system and (4) information campaigns related to the zoonotic risk of *M. bovis* in rural areas.

## RÉSUMÉ

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En Equateur, comme dans d'autres pays d'Amérique Latine, la tuberculose bovine reste un problème important tant au niveau de la santé animale que de la santé humaine. L'OMS considère comme prioritaire les programmes de lutte contre la tuberculose en Amérique latine. La spéculation laitière dans les zones péri-urbaines associée à l'absence de programmes de contrôle officiels de la tuberculose bovine représentent des facteurs de risque majeurs en Equateur. Il existe très peu de données sur l'importance de la tuberculose bovine dans les élevages laitiers des régions andines et, jusqu'à présent, aucun laboratoire n'était compétent pour le diagnostic des mycobactéries d'origine animale. C'est dans ce contexte que ce projet a été initié. Les objectifs de cette thèse étaient (i) de fournir des données sur la situation épidémiologique de la tuberculose bovine dans les élevages laitiers du nord de l'Equateur et, (ii) de comparer les performances des outils de diagnostic classiques utilisés pour identifier *Mycobacterium bovis* (*M. bovis*).

Une première étude a été réalisée dans le canton de Mejia en 2003. Les campagnes de tuberculation (intradermo-tuberculation simple [IDS] et double [IDD]) ont concerné plus de 1.000 bovins provenant de 59 troupeaux classés en 3 catégories selon leur taille : catégorie 1 (> 70 têtes) ; catégorie 2 (entre 25 et 70 têtes) et catégorie 3 (< 25 têtes). Au sein des troupeaux de grande taille, 7.95 % de bovins réagissaient positivement aux tests cutanés alors qu'en catégorie 2 et 3, respectivement 4.24 % et 0.30 % des bovins réagissaient positivement. Cette première enquête a permis de mettre en évidence la présence de *M. bovis* dans les élevages laitiers du canton de Mejia avec une prévalence plus importante dans les troupeaux de grande taille.

Une enquête plus détaillée a été réalisée en 2007 et 2008 dans la même région. Au total, plus de 2.000 bovins issus de 20 troupeaux ont été investigués. En 2007, chaque animal a été testé (IDD) et un suivi a été réalisé dans les mêmes troupeaux l'année suivante. L'incidence annuelle réelle était de 1.70 %. Au cours des 2 années, des différences significatives ont été mises en évidence entre les prévalences calculées dans les troupeaux de taille moyenne (0.27 % et 0.57 %) et celles calculées dans les troupeaux de grande taille (8.63 % et 8.43 %). La taille du troupeau a clairement été identifiée comme un facteur de risque pour la tuberculose bovine, de même que l'âge des bovins, le contact éventuel avec d'autres espèces animales et l'introduction de nouveaux bovins dans l'exploitation.

Une troisième étude a été menée dans un abattoir du canton de Mejia sur 1390 bovins abattus sur une période de deux ans. Au total, 33 bovins ont été détectés avec des lésions macroscopiques suspectes, soit 2.3 % et 2.4 % des animaux inspectés en 2007 et 2008. Parmi ces 33 cas suspects, des bacilles acido-résistants ont été mis en évidence dans 33% des cas (microscopie directe) et des lésions microscopiques suggestives dans 27 % des cas (histopathologie). La mise en culture et la PCR ont mis en évidence la présence de *M. bovis* dans 36 % et 27 % des cas suspects, respectivement. L'utilisation combinée de ces 4 outils de diagnostic a permis de confirmer que 51 % des animaux suspects étaient positifs pour *M. bovis*. Comparés à la mise en culture, considérée comme la technique de référence pour la détection de *M. bovis*, les autres outils de diagnostic présentaient une sensibilité de 56 % (PCR) et 43 % (microscopie et histopathologie) et une spécificité de 94 % (PCR et microscopie) et 97 % (histopathologie). Ces résultats soulignent que plusieurs outils doivent être utilisés en parallèle pour le diagnostic *post mortem* de la tuberculose bovine. Les lésions de tuberculose étant principalement localisées au niveau des ganglions thoraciques, la



transmission de *M. bovis* par voie aérogène semble la plus plausible pour les bovins inclus dans cette enquête.

Pour compléter cette troisième étude, une approche par spoligotypage a été mise en œuvre. Toutes les souches (n = 23) isolées à l'abattoir présentaient le même profil (spoligotype SB0980). Une étude d'envergure devrait être envisagée afin de déterminer la diversité des souches de *M. bovis* présentes en Equateur.

Dans les pays qui ne possèdent pas de politique de contrôle de la tuberculose bovine dans les élevages, *M. bovis* peut représenter un problème de santé publique. La cinquième étude avait pour objectif de déterminer la prévalence de *Mycobacterium* spp dans des populations humaines ciblées. Des tests cutanés ont été réalisés sur 157 personnes appartenant à deux groupes à risque (personnel d'abattoir et techniciens d'élevage). Au total, 29 % des personnes testées présentaient une réaction positive au test cutané. Les facteurs de risque majeurs associés aux résultats cutanés positifs étaient la consommation de lait cru et de fromages confectionnés à la main. Des campagnes d'information en zone rurale sont nécessaires pour conscientiser la population au risque zoonotique de la tuberculose bovine.

En conclusion, les données présentées dans ce manuscrit soulignent l'importance de *M. bovis* dans les élevages de bovins laitiers dans le canton de Mejia. Une politique de contrôle de la tuberculose bovine devrait être envisagée à l'échelle nationale. Idéalement, ce programme devrait inclure (1) un laboratoire de référence pour les mycobactéries et des formations spécialisées pour les inspecteurs vétérinaires; (2) une procédure obligatoire de détection-abattage des bovins infectés, avec compensation financière; (3) des procédures d'inspection *post mortem* standardisées et associées à des analyses de laboratoire et un système de

traçabilité et enfin, (4) des campagnes d'information en zones rurales relatives au risque zoonotique associé à *M. bovis*.

## RESUMEN

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En Ecuador, como en otros países latinoamericanos, la tuberculosis bovina (TBB) es un problema importante en la industria lechera, con implicaciones económicas y en la salud pública. El aumento de la producción lechera en áreas periurbanas asociada a la ausencia de un programa de control de TBB en Ecuador son factores de riesgo importantes en el contexto de la TBB. Complementariamente, los datos oficiales relacionados con la enfermedad son escasos, y no existe en Ecuador un laboratorio veterinario de referencia nacional para el estudio de micobacterias; con este contexto el presente trabajo fue planificado. Los objetivos principales fueron proporcionar información de la situación epidemiológica de la TBB en el ganado lechero del norte del Ecuador, y comparar el funcionamiento de las herramientas estándar de diagnóstico utilizadas para identificar *M. bovis*.

En un primer paso, en el año 2003 se desarrollo un estudio en el cantón de Mejía, una de las principales regiones lecheras del norte del país, utilizando pruebas de tuberculinización. Aleatoriamente se seleccionó 1012 bovinos provenientes de 59 fincas lecheras, clasificados según el tamaño del hato, estos animales fueron sometidos a las pruebas de tuberculinización intradérmica simple (TIS) y tuberculinización intradérmica comparativa (TIC). Los resultados fueron 7.95% de reactores positivos en hatos grandes, en contraste con 4.24% en medianos y 0.30% en pequeños hatos. Estos primeros resultados evidencian la importancia de la TBB en el ganado lecheros del cantón de Mejía e identifican al tamaño del hato como un factor de riesgo.

Posteriormente, se realizó una investigación más detallada en la misma región durante los años 2007 y 2008, en un total de 2022 bovinos provenientes de 20 hatos lecheros. En el 2007, cada animal fue diagnosticado a través de la prueba de TIC y un año después se realizó un seguimiento utilizando la misma prueba en el mismo grupo de animales; la incidencia real anual fue calculada en 1.70%. El análisis de los resultados de las pruebas de TIC demostraron una diferencia significativa entre hatos lecheros medianos y grandes, con una prevalencia de TBB de 0.27% y 0.57% en hatos medianos, comparados con el 8.63% y 8.43% en hatos grandes para los años 2007 y 2008, respectivamente. El tamaño del hato fue identificado como un factor de riesgo significativo, confirmando los resultados del primer estudio. El número de reactores positivos a la prueba TIC también aumentó considerablemente con la edad, el contacto con otras especies de animales y con la introducción de nuevos animales en el hato.

Un tercer estudio fue realizado en 1390 bovinos sacrificados durante un período de dos años, en el se comparó de las pruebas de diagnóstico clásicas para detectar ganado infectado. Un total de 33 bovinos con lesiones evidentes (sospechosas) fueron detectados, representando un 2.3% y 2.4% de los animales examinados en 2007 y 2008, respectivamente. Del total de casos sospechosos, solamente fueron identificados bacilos ácido-resistentes (microscopia directa) en 33% (11/33) de los casos, y a través de las lesiones microscópicas sugestivas a TBB (histopatología) en 27.3% (9/33). El cultivo *in vitro* y PCR detectaron el 36.4% de animales positivos (12/33) y el 27.3% (9/33), respectivamente. Al utilizar de manera combinada todas las pruebas para la identificación de animales infectados (microscopia, histopatología, PCR y cultivo *in vitro*), en los cuales por lo menos una prueba era positiva, se obtuvo el 51.5% (17/33) de bovinos infectados del total de animales sospechosos. Comparando el cultivo *in vitro* con otras pruebas de laboratorio, se calculó la sensibilidad (Se) y especificidad (Sp) de

las otras pruebas, la PCR tuvo una Se de 56.5%, la microscopia e histopatología 43.5%; mientras que la Sp para la PCR y microscopia fue de 94.4% y 97.2% para la histopatología. Estos resultados demuestran que un diagnóstico post-mortem confiable requiere de la combinación de varias pruebas de laboratorio, es necesario el desarrollo y mejoramiento de herramientas de diagnóstico que brinden mejores características de funcionamiento. Tomando en cuenta que las lesiones macroscópicas fueron observadas principalmente en ganglios linfáticos torácicos, se sugiere que la vía respiratoria es la principal ruta para la transmisión de la TBB en ganado lechero examinado en el camal del cantón Mejía.

En un paso adelante, se utilizó el spoligotyping para comparar las cepas de *M. bovis*. Todas las cepas aisladas (n = 23) del ganado sacrificado del cantón de Mejía presentaron el mismo spoligotipo (SB0980); tal homogeneidad podía estar relacionada con un área de estudio relativamente pequeña. Un estudio de epidemiología molecular más extenso, a un nivel nacional, podía ser provechoso para determinar la diversidad de las cepas de *M. bovis* en la población bovina del Ecuador. En países donde no existe control de la TBB, *M. bovis* es también de interés de la salud pública.

El último estudio fue planificado para evaluar la prevalencia de *Mycobacterium* spp. en la población humana en riesgo a través de la prueba de tuberculinización. El estudio fue conducido en 157 personas (trabajadores de finca y camal), la prueba demostró una alta prevalencia de reactivos (29%). Los principales factores de riesgo asociados a la reactividad en humanos fueron el consumo de leche cruda y queso artesanal. Con estos resultados se hace necesaria la implementación de campañas de información, para difundir en las poblaciones rurales el riesgo zoonótico relacionado con *M. bovis*.

En conclusión, los datos presentados en esta tesis confirman la importancia de la TBB en el ganado lechero del norte del Ecuador y destacan la necesidad de un programa nacional de control de la TBB. Preferentemente, este programa debe incluir (1) la existencia de un laboratorio de referencia para la TBB y el entrenamiento de veterinarios calificados; (2) pruebas tuberculínicas obligatorias y compensación oficial por animales reactivos; (3) inspección post-*mortem* detallada con diagnóstico de laboratorio asociados a un sistema de trazabilidad de animales tuberculosos, y (4) campañas de información relacionadas con el riesgo zoonótico de *M. bovis* en zonas rurales.

## LIST OF ABBREVIATIONS

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**AD:** *Anno Domini*

**AFB:** Acid fast bacilli

**BCG:** Bacille Calmette-Guérin

**bp:** Base pair

**BTB:** Bovine tuberculosis

**CD4+:** Cluster of differentiation 4 glycoprotein

**CDS:** CoDing Sequence

**CFSPH:** The Center for Food Security and Public Health

**CITT:** Comparative intradermal tuberculin test

**DNA:** Desoxyribonucleic acid

**DR:** Direct repeat

**ELISA:** Enzyme-linked immunosorbent assay

**ESAT-6:** 6 kDa early secretory antigenic target

**FAO:** Food and Agriculture Organization

**FDA:** Food and Drug Administration

**GC:** Guanine - cytosine

**G+C:** Guanine plus cytosine

**GM-CSF:** Granulocyte monocyte colony stimulating factor

**HIV:** Human immunodeficiency virus

**$\gamma$ -IFN:** Gamma interferon



**IL:** Interleukin

**INPAAZ:** Pan American Institute for Food Protection and Zoonoses

**IS:** Insertion sequence

**IU:** International Units

**MGIT:** Mycobacteria Growth Indicator Tube

**MIF:** Migration inhibition factor

**MIRU:** Mycobacterial interspersed repetitive units

**MOTT:** Mycobacteria other than tuberculosis

**NTM:** Non-tuberculous mycobacteria

**OIE:** Office International des Epizooties

**OR:** Odds ratio

**OT:** Old tuberculin

**PAHO:** Pan American Health Organization

**PANTA:** Polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin

**PCR:** Polymerase chain reaction

**PGRS:** Polymorphic guanine-cytosine rich sequences

**PPD:** Purified protein derivative

**PZA:** Pyrazinamide

**rDNA:** Ribosomal desoxyribonucleic acid

**RFLP:** Restriction fragment length polymorphism

**RNA:** Ribonucleic acid

**rRNA:** Ribosomal ribonucleic acid

**Se:** Sensitivity

**SITT:** Simple intradermal tuberculin test

**Sp:** Specificity

**ST:** Spoligotype

**TB:** Tuberculosis

**TCH:** Thiophene-2-carboxylic acid hydrazide

**Th1:** T helper 1 lymphocyte

**TST:** Tuberculin skin test

**UK:** United Kingdom

**UV:** Ultraviolet

**VNTR:** Variable number tandem repeats

**WHO:** World Health Organization

**ZN:** Ziehl Neelsen

# **CHAPTER 1 – INTRODUCTION**

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## 1 INTRODUCTION

### 1.1 *History of bovine tuberculosis*

BTB among cattle was first described in 14 A.D. by Columella in Northern Italy (Wood *et al.*, 1994). However, the earliest documentation of a macroscopically recognized TB infection was carried out in a fossil from an extinct Bison (*Bison antiquus*), dated 17.000 years ago. According to this study, bovids from North America were the vectors that transported the causative agent of BTB. DNA-based identification of this *M. tuberculosis*-complex strain showed 72.7% similarity with the current *M. bovis* (Rothschild *et al.*, 2001). Initially, it has been suggested that *M. tuberculosis* arose from *M. bovis* at the time of the domestication of cattle, approximately 10 to 15,000 years ago. This was based on the observation of the wide host range of *M. bovis*, infecting wild and domesticated mammals as well as man, whereas *M. tuberculosis* appears to be restricted mostly to humans (Hewinson *et al.*, 2006). However, recent DNA analyses contradict this hypothesis, the systematic analysis of polymorphisms in a large panel of strains indicate that *M. canettii* is likely the ancestral specie of the *M. tuberculosis* complex (Thoen & Barletta, 2006).

In 1865, a French military doctor, Jean Antoine Villemin, demonstrated that “consumption” or tuberculosis (TB) was an infectious disease from humans and cattle. He made experiments inoculating laboratory rabbits with material from infected humans and cattle, and published his results in the treatise “*Etudes sur la Tuberculosis*” (Studies on Tuberculosis), which describes the transmission of TB from humans to rabbits, from cattle to rabbits, and from rabbits to rabbits. He suggested that a specific microorganism could be the cause of the disease that could be transmitted from human to cattle (Villemin, 1868).

In 1882, Robert Koch demonstrated that TB was caused by tubercle bacilli and performed cutaneous inoculations in guinea pigs. He developed a staining method to identify acid-fast bacilli and cultured them on solid medium. Koch also isolated a factor from culture filtrates which halted the growth of TB bacilli, known as tuberculin and now described as Koch's old tuberculin (OT). This was inoculated and used to diagnose TB in man since 1890. It was the first clear demonstration of delayed hypersensitivity or cell-mediated immunity. The technique was adapted by veterinarians and used for testing cattle mainly through the febrile reaction in tuberculous cows. (Daniel, 2005; Koch, 1906).

In 1898, an American researcher, Theobald Smith, observed differences between tubercle bacilli of bovine and human origin, and named them as the human and bovine variants of the tubercle bacillus, even though he warned against the assumption that these strains were limited to their respective hosts (Smith, 1898; Grange, 2001). However, only since 1911, the Royal Commission named by the British Government had firmly established that the human population was susceptible to BTB and laid the foundations of the test-slaughter policy for BTB eradication (Anonymous, 1911).

In 1921, two French bacteriologists: Albert Calmette and Camille Guérin (veterinarian) investigated the intestinal route of TB infection. Calmette and Guérin began to grow *M. bovis* in a beef bile-glycerine medium, with continuous replanting of the culture in this medium, and thus lowering the virulence of *M. bovis* strain. This attenuated strain was called "Bacille-Calmette-Guérin" (BCG). Exhaustive testing of BCG showed its safety and effectiveness in protecting young animals against TB. In 1924, the vaccination of newborn infants with BCG began in France to protect against severe forms of TB, but its effect did not prevent

pulmonary TB in adults. The BCG vaccine was spread worldwide and it is still widely used today in developing countries (Hawgood, 2007; Oettinger *et al.*, 1999).

## 1.2 Taxonomy and characteristics of the *Mycobacterium bovis*

*M. bovis* is one of the members of *M. tuberculosis* complex. Current taxonomy recognizes 8 members in this group i.e., *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. caprae*, *M. africanum*, *M. pinnipedii*, *M. microti* and *M. cannettii* (Wayne & Kubica, 1986; van Soolingen *et al.*, 1997; Pfyffer *et al.*, 1998; Cousins *et al.*, 2003; Aranaz *et al.*, 2003).

The genus *Mycobacterium* is the unique genus of the family Mycobacteriaceae, order Actinomycetales, class Actinomycetes. Apart of the *M. tuberculosis*-complex the genus *Mycobacterium* comprises *M. leprae*, *M. ulcerans* and more than 100 species of non-tuberculous mycobacteria (NTM) or mycobacteria other than tuberculosis (MOTT). The latter are distributed in four groups according to the Runyon classification, i.e., group I = slow growers – photochromogen, group II = slow growers – scotochromogen, group III = slow growers – nonchromogen, and group IV = rapid growers (Wayne & Kubica, 1986).

**Table 1: Members of the *Mycobacterium tuberculosis* complex and hosts**

	<b>Members</b>	<b>Main hosts</b>
<i>M. tuberculosis</i> Complex	<i>M. tuberculosis</i>	Human TB
	<i>M. bovis</i> , <i>M. bovis</i> BCG	Cattle and human TB
	<i>M. africanum</i>	Human TB in Africa
	<i>M. canettii</i>	Human TB
	<i>M. microti</i>	TB in small rodents
	<i>M. caprae</i>	TB in goats
	<i>M. pinnipedii</i>	TB in seals

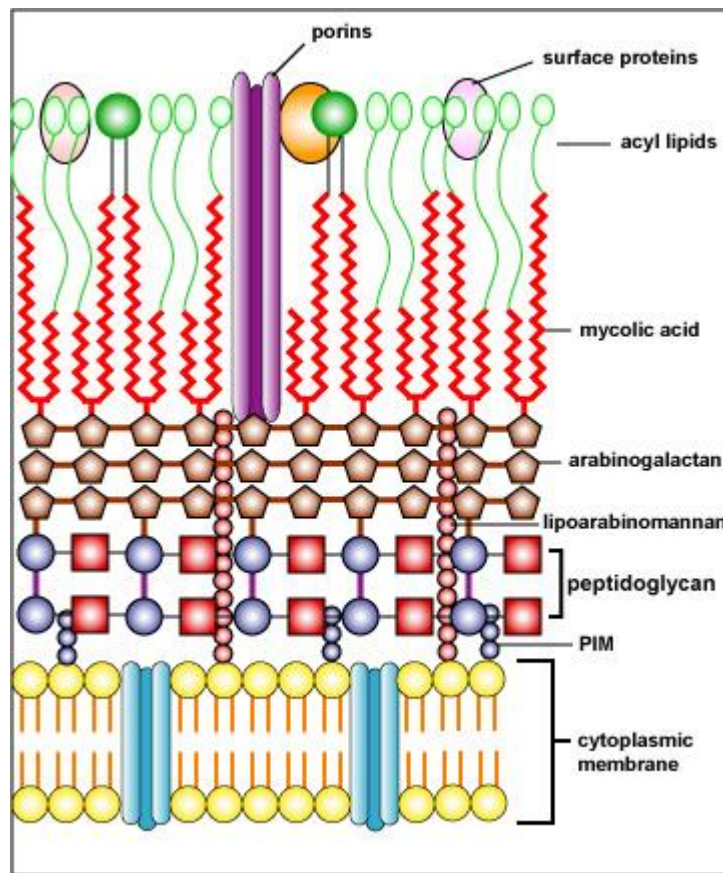
Tubercle bacilli are aerobic and predominantly rod-shaped, 0.3 to 0.5µm wide and with variable length (1.5 – 3.5 µm). Spores, flagella and capsules are absent. It is an acid-fast bacillus, an important characteristic that has been taken advantage for Ziehl Neelsen staining. The bacillus resists decolourization with acid-alcohol after a first red stain with carbol fuchsin, and can be easily detected when using a methylene blue counterstain (Biberstein & Hirsh, 1999). Although cytochemically Gram-positive, mycobacteria do not take up the dyes of the Gram-stain because the cell walls are rich in lipids and mycolic acids (Quinn *et al.*, 1999).

Tubercle bacilli are rather resistant to disinfectants and require long contact times for its inactivation. They survive exposure to alkali or acids for 15 to 30 minutes, conditions commonly used to decontaminate diagnostic specimens (Portaels *et al.*, 2001). They resist drying and survive for long periods in soil, but are fairly heat sensitive and killed by sunlight, ultraviolet irradiation and pasteurization (Biberstein & Hirsh, 1999).

Mycobacteria are most closely related to the genus *Nocardia* and *Rhodococcus* and all three genres have a similar cell wall type (Quinn *et al.*, 1999). The genus *Mycobacterium* has a unique cell wall formed by abundant lipids, which have a low permeability. These lipids account for acid fastness, as well as pathogenic and immunologic properties. The wall presents a double layer of peptidoglycan and arabinogalactan surrounded by long chain mycolic acids (Figure 1). Mycosides, phospholipids and sulpholipids apparently protect tubercle bacilli against phagocytic killing and allow intracellular and extracellular multiplication to continue. The cell wall is the interface between the bacillus and host; many



pathogenic genes are encoding cell wall structures and secreted proteins (Hewinson *et al.*, 2006).



**Figure 1. Structure of the mycobacterial cell wall.**

Mycobacteria produce a thick mycolate-rich outer covering which functions as an exceptionally efficient barrier.

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Updated: July, 2008

### 1.2.1 Characteristics of *M. bovis*

Although *M. bovis* is an obligate intracellular pathogen, experimental studies have shown that it can survive under specific conditions. The survival rates are different depending on the

availability of nutrients, and climatic conditions i.e., humidity, exposure to sunlight and temperature (Morris *et al.*, 1994). *M. bovis* can survive in faeces, blood, and urine from 150 to 332 days at 12 to 24°C, when shielded from direct sunlight, particularly in cold, dark and moist conditions (Genov, 1965). Nevertheless, in natural pastures *M. bovis* remained viable and virulent for at least 7 weeks during the English summer (Maddock, 1933). Under artificial conditions, *M. bovis* can be cultured from stored samples after two years, which were kept at -20°C in tubes containing Dubos broth supplemented with PANTA to inhibit the growth of contaminants and to preserve the mycobacteria (Proaño-Perez *et al.*, 2009). In some dairy products (i.e., cheese), tubercle bacilli can survive for up to 14 days after processing (Gallagher & Jenkins, 1998).

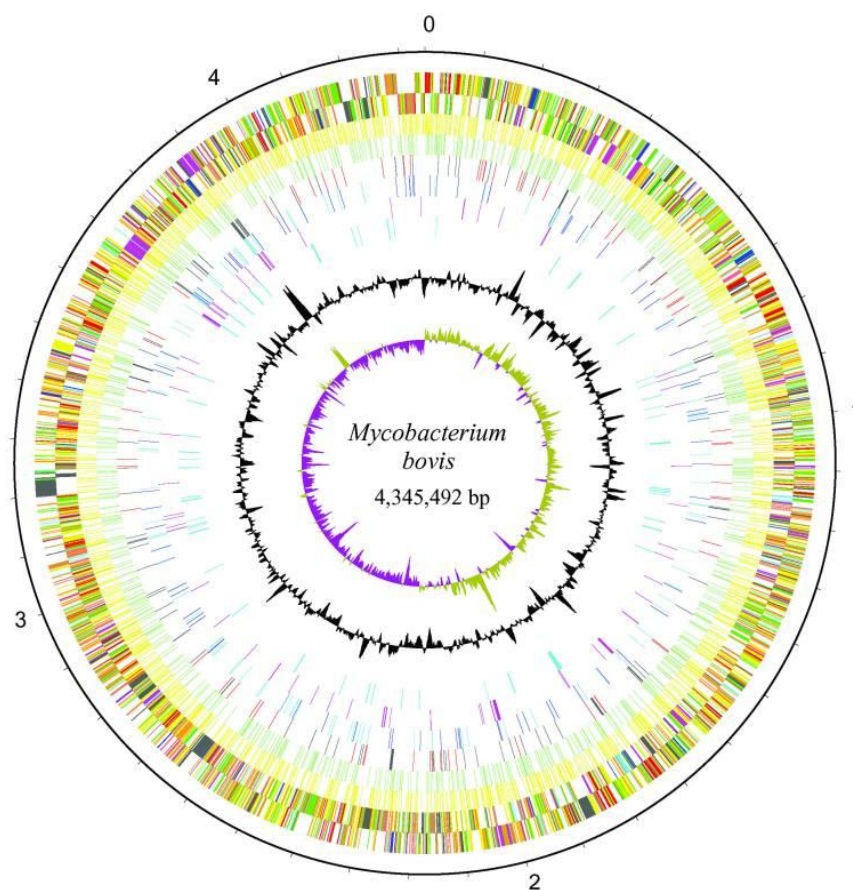
*M. bovis* strains display “dysgonic” growth on media containing glycerol. Therefore, the bacillus requires pyruvate to be added to media where glycerol is the sole carbon source. This reflects a defect in the metabolism of glycerol, due to a mutation in the gene for the pyruvate kinase enzyme, which catalyses the final step in the glycolysis (Keating *et al.*, 2005). *M. bovis* is intrinsically resistant to pyrazinamide (PZA), due to the lack of enzyme pyrazinamidase, which converts the PZA to the toxic pyrazinoic acid (Konno *et al.*, 1967). Consequently, PZA cannot be used as a drug for TB treatment caused by *M. bovis*.

### **1.2.2 *M. bovis* genome**

The completion of a number of mycobacterial genome sequences can allow the development of antigen mining techniques that rapidly identify *M. bovis*-specific genes. The genome contains approximately 4000 genes, encoding different properties, potential virulence factors and antigens (Hewinson *et al.*, 2006). Cell wall components and secreted proteins show the

greatest variation compared with *M. tuberculosis* and *M. leprae*, indicating their important role in host-bacillus interactions or immune evasion. These variations were the key to the evolution of *M. bovis* (Garnier *et al.*, 2003).

Figure 2 shows the circular representation of the *M. bovis* genome in comparison with that of the *M. tuberculosis* H37Rv reference strain. The *M. bovis* genome is > 99.95% identical at the nucleotide level to that of *M. tuberculosis*, showing colinearity and no evidence of extensive translocations, duplications or inversions.



**Figure 2. Circular representation of the *M. bovis* genome** (Garnier *et al.*, 2003).

The scale is shown in megabases by the outer black circle. Moving in from the outside, the next two circles show forward and reverse strand CDS, respectively, with colors representing the functional classification. Comparisons with the *M. tuberculosis* H37Rv sequence are then shown, with transitions (yellow) and transversions (green), then insertions (red, 1 bp; black >1 bp) and deletions (dark blue, 1 bp; light blue >1 bp); sequence replacements by novel regions in *M. bovis* are then shown (purple). IS elements and phage (cyan) are displayed in the following circle, with G+C content and then finally GC bias (G-C)/(G+C) shown by using a 20-kb window.

### **1.3 Pathogenesis of *M. bovis*.**

Pathogenesis is the process or mechanism of disease. In the case of BTB this process comprises several elements: sources and routes of infection, intrinsic properties of the bacillus, and interaction with the host and immune responses. Both humoral and cell-mediated immune responses can be induced after mycobacterial infection. However, the cell-mediated immune system has the most significant role in protective immunity against *M. bovis* (Neill *et al.*, 1994).

Tubercle bacilli do not produce toxins and their pathological effects result essentially from the strong antigenicity of their cell walls (Gallagher & Jenkins, 1998). Virulence of *M. bovis* appears to reside in the lipids of the cell wall, i.e., mycosides, phospholipids and sulpholipids, which are also thought to protect the tubercle bacilli against phagocytosis. In the first week after infection, cell-mediated immune reactions begin to modify the host response. Infected macrophages secrete IL-12, a cytokine responsible for stimulating CD4<sup>+</sup>T<sub>H1</sub> lymphocytes to produce gamma interferon (IFN- $\gamma$ ), granulocyte monocyte colony stimulating factor (GM-CSF), and migration inhibition factor (MIF), which attract and activate macrophages. Activated macrophages acquire the capacity to kill some mycobacteria, and their efficiency depends on the adequacy of the immune response and the virulence of bacteria. The aggregation of macrophages contributes to the formation of a tubercle (Biberstein & Hirsh, 1999; Quinn *et al.*, 1999).

The primary lung lesion is a bronchopneumonic focus which may progress rapidly or may remain quiescent for many years (Morris *et al.*, 1994). Once cell-mediated immunity is established, lymphatic spread is retarded but contiguous extension via the erosion of bronchi,

blood vessels or viscera to new areas, and T-lymphocyte-mediated reactions cause tissue damage (Quinn *et al.*, 1999). Immature and aged individuals often develop more severe lesions than mature animals. Zebu cattle are more resistant than European breeds (Biberstein & Hirsh, 1999).

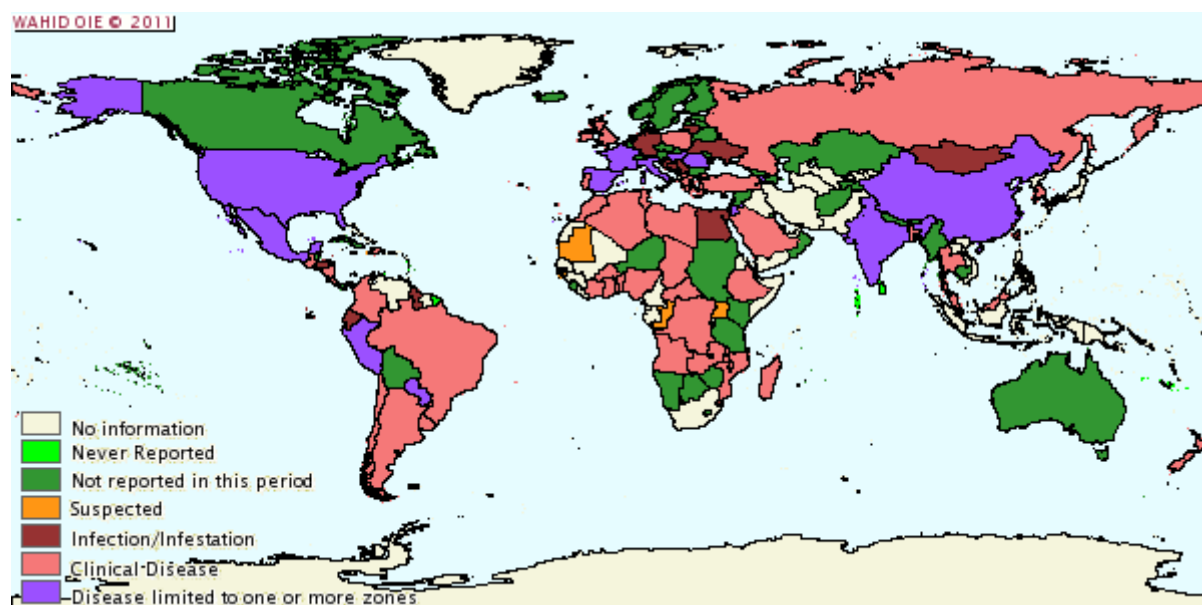
#### **1.4 Epidemiology of bovine tuberculosis**

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis* is a worldwide distributed disease, with economic and public health importance. In humans, BTB is being considered as a re-emergent zoonosis related to different situations: the persistence of the infection in livestock, the maintenance of wildlife reservoirs, the appearance of strains resistant to the main anti-TB drugs, and/or the spread of the human immunodeficiency virus (Cosivi *et al.*, 1998; Abalos & Retamal, 2004). In developing countries from Africa, Asia, Latin America and the Caribbean, persistence or increase of BTB among cattle is caused by the increasing production in the dairy industry, intensification of dairy herds, and the lack of appropriate measures to control the disease (Suazo *et al.*, 2003).

##### **1.4.1 Prevalence of BTB in cattle**

In many industrialised countries, BTB has been controlled or eradicated by application of tests and slaughter programmes. Nevertheless, sporadic cases are still found among cattle in some of these countries like the United Kingdom (UK) and Ireland, where the presence of *M. bovis* in wildlife reservoirs seems to be the main cause of persistence of BTB (Phillips *et al.*, 2003). Several countries have been officially classified as tuberculosis-free i.e., Australia, Iceland, Denmark, Sweden, Norway, Finland, Austria, Switzerland, Luxembourg, Latvia,

Slovakia, Lithuania, Estonia, the Czech Republic, Singapore, Jamaica, Barbados and Israel (CFSPH, 2007). Figure 3 shows the distribution of BTB among cattle in the world in 2010, classified according to the occurrence of the disease.



**Figure 3. Distribution of bovine tuberculosis among cattle based on data obtained between January and June 2010. (OIE, 2010)**

In Latin America, the presence of BTB among cattle is not well quantified (de Kantor & Ritacco, 2006). Out of thirty four Latin American and Caribbean countries, twelve reported BTB as sporadic or with low occurrence, seven have reported BTB as an enzootic disease, and only in one country (Dominican Republic) a high occurrence was noticed. On the other hand, BTB was absent in twelve countries and data were lacking in the remaining two. Control measures based on the test-and-slaughter policy have been established in only 12 countries in the region that consider BTB as a notifiable disease. In the remaining 22 nations, the disease is only partly or not at all controlled (Cosivi *et al.*, 1998).

About 70% of the cattle population in Latin America and the Caribbean (261 million) is held in areas where *M. bovis*-infection rates are higher than 1%. Ecuador, together with Haiti, Guatemala, Argentina, Bolivia, Brazil, Chile, Peru and Guyana, are considered to belong to a group of countries with a relatively high prevalence or no officially reported information (de Kantor & Ritacco, 2006).

In Mexico, like in other developing countries the occurrence of BTB is much higher in dairy than in beef cattle. The prevalence of BTB in beef cattle has been estimated at 2.9% (Zendejas-Martinez *et al.*, 2008), while in dairy cattle it was approximately 16%. The high prevalence observed in this study, is thought to be caused by the lack of farmers' participation in the eradication measures (Perez-Guerrero *et al.*, 2008). The disease is affecting dairy cattle from circumscribed areas. In Queretaro, 17% of gross TB lesions were found among 1201 carcasses (Milián-Suazo *et al.*, 2000). According to Zendejas-Martinez *et al.*, (2008), at least two reasons can explain this observation, i.e., management practices and food supply. Dairy cattle are maintained under intensive production conditions, with large numbers of animals kept per unit area, which increases the risk of disease transmission by the close proximity between cattle. Dairy herds are mainly kept close to urban areas. On the contrary, beef cattle are raised in dry coastal areas, with a limited number of cattle per unit area, conditions reducing disease transmission and survival of the bacillus.

In Argentina, since a test and culling policy to control and eradicate BTB was put in force in 1999, 7713 farms have been officially declared free of infection, of which 6767 are dairy farms. This measure has decreased the prevalence of TB in cattle and swine. Nowadays, the national rates of BTB in cattle and swine detected by the observation of macroscopic lesions in abattoirs have been reduced from 6.7% and 8.4% in 1969, to 0.9% and 0.4% in 2008,



respectively (Torres, 2009). TB in swine has been controlled by farming improvements including slaughter at an earlier age and avoiding the practice of feeding animals with non-pasteurised dairy products (Perez *et al.*, 2002). At slaughterhouses, animals with lesions are confirmed by laboratory diagnosis, followed by tracebacking the origin of infected animals and tuberculin tests are applied in the herds (Torres, 2009).

In Brazil, an average of 7.1% of herds were infected with BTB as estimated during a tuberculin testing survey in 1998, ranging from 2.8% in the Central West to 58.3% in the Northern regions. TB lesions have been observed in 0.07% of 9.5 million cattle slaughtered between 1993 and 1997 in Minas Gerais (de Kantor & Ritacco, 2006); while in Mato Grosso do Sul, only 0.2% of lesions suggestive of BTB were detected by veterinary inspection (Pires de Araujo *et al.*, 2005). Nevertheless, a high BTB prevalence was reported in a dairy area close to Rio de Janeiro, with 12.7% skin test reactors (Lilenbaum *et al.*, 1999).

In Colombia, no data on the prevalence of BTB are publically available. Tuberculin skin testing and culling of all positive animals is the official preventive measure in place, but BTB is not yet under control. (Romero *et al.*, 1999).

In Ecuador, the national prevalence of BTB is unknown and the scarce information available is restricted to tuberculin testing surveys performed in only some areas of the country. In absence of a national BTB control program is difficult to organize the data, apply sanitary regulations or propose measures to control the disease, and to estimate its effects. The expansion of the dairy industry caused by the high demand for milk and byproducts, the intensification of the farms, an increase of the cattle population, the presence of *M. bovis* and the lack of control measures are influencing the raise in BTB prevalence in Ecuador. In 1977,

the first survey conducted to identify the prevalence of BTB on dairy cattle showed a low occurrence of 0.33% (Acosta & Parreño, 1977), whereas in 2003, the prevalence increased to 1.22% (Aleman *et al.*, 2003) in the same area (Tungurahua province). In 2008, the true annual incidence was estimated at 1.70% and the true prevalence at 7.13%, in medium and large herds from the Mejia canton, an important dairy region in the north of Ecuador. In addition, 2.24% of cattle were diagnosed at slaughtering, and the risk factors of BTB among dairy cattle in this region were identified, namely herd size, age, contacts with other animal species, and introduction of new cattle (Proaño-Perez *et al.*, 2006; Proaño-Perez *et al.*, 2009).

#### **1.4.2 Transmission of BTB among cattle**

Cattle to cattle transmission mainly occurs by the respiratory route (80-90%), whereas the digestive tract is an important route of transmission between species (Morris *et al.*, 1994; Goodchild & Clifton-Hadley, 2001). According to Gil & Samartino (2001), cattle are resistant to *M. avium* infections and more yet to *M. tuberculosis*; however, the presence of *M. avium* can influence the skin tests in exposed cattle.

In dairy farms, the intensive management system can increase the rate of cattle to cattle transmission in the herd, and the head to head proximity during feeding or drinking can facilitate the spread of the bacillus (Goodchild & Clifton-Hadley, 2001). Mycobacteria can be excreted in sputum, milk, urine, or faeces and contaminate the environment, leading to infections of animals and man (Phillips *et al.*, 2003). In aerogenous infections, bacteria are inhaled in droplets nuclei of 2 to 5 µm in diameter which have the ability to reach the alveolar spaces of the lungs; the bacteria contained in the droplet are ingested by macrophages. Larger particles (or nuclei) are trapped in the upper airways and are removed by means of the

mucocilliary apparatus, after which they are swallowed (Cousins *et al.*, 2004). Infection acquired through ingestion of *M. bovis* is more likely to result in non-pulmonary forms of the disease (Cosivi *et al.*, 1998).

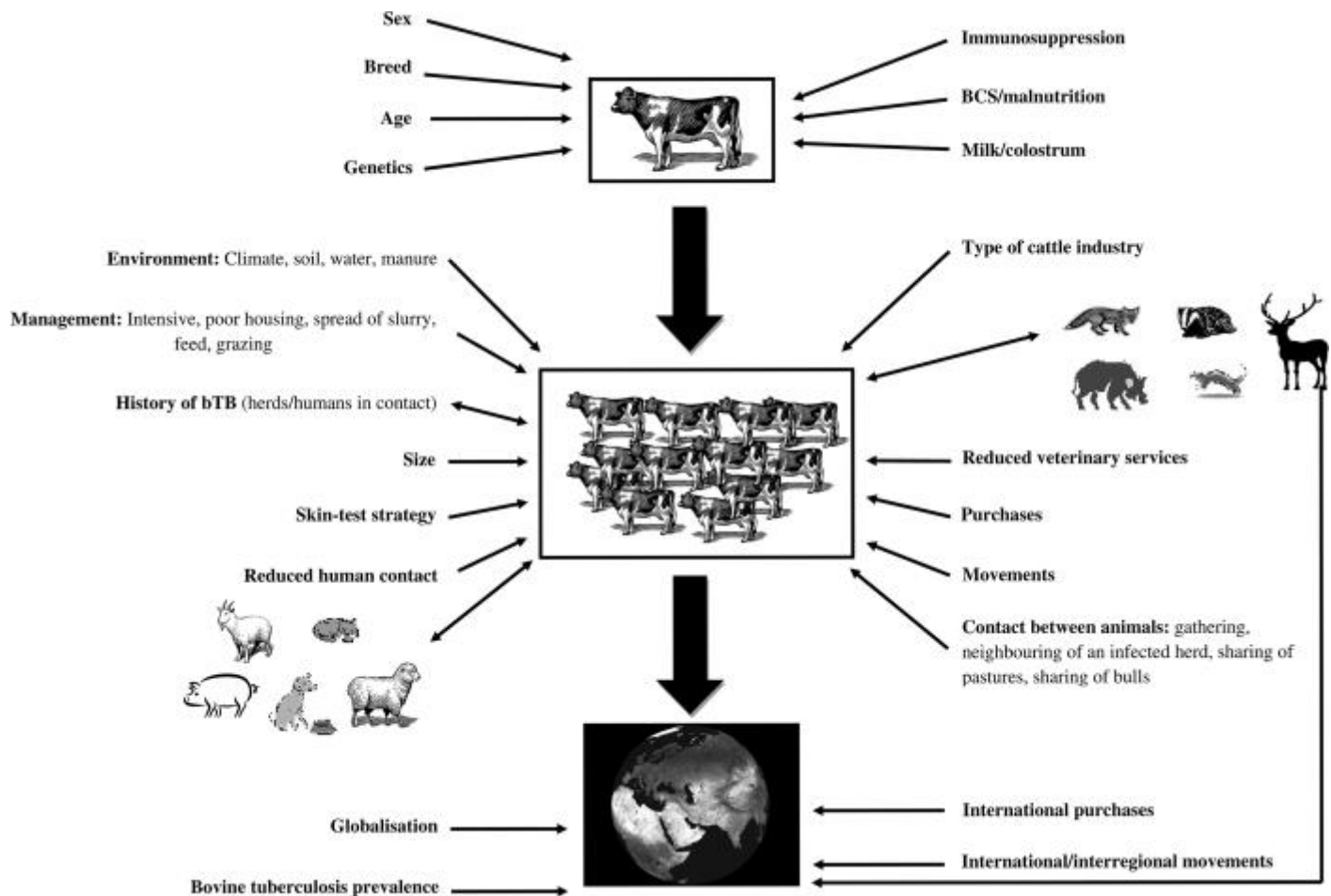
Cattle remain asymptomatic for some months or, in some cases, infection can be latent for years before to become active. Commonly the disease appears when the balance between the host and the infectious agent is disrupted during periods of stress or in old cattle (Acha & Szyfres, 2001). The period of latency precedes the period in which animals can be infectious to others, during this period animals do not react to skin tests; the length of the unresponsive period can vary between 8 and 65 days (Goodchild & Clifton-Hadley, 2001). It was estimated that in the absence of control measures, one infectious animal infects 2.2 cattle per year (in average) in an Argentinean dairy farm with semi-extensive farming conditions. This rate was calculated taking into account that BTB is a chronic disease using a mean incubation period of 24 months (Perez *et al.*, 2002).

Vertical transmission can also occur in infected females. The uterus may serve as a portal for foetal infection and surviving calves commonly develop liver and spleen lesions (Biberstein & Hirsh, 1999). Nevertheless, only 1% of calves from tuberculous cows can be congenitally infected (Morris *et al.*, 1994). Another possibility for infection from cow to calf is by ingestion of colostrum or milk contaminated with *M. bovis* (Zanini *et al.*, 1998). The large doses needed for infection by oral route in cattle explains the low infectivity of *M. bovis*-contaminated faeces (Morris *et al.*, 1994). However, the risk of infection by this way may be important in developing countries where control measures are not effective (Humblet *et al.*, 2009).

Human beings with open TB due to *M. bovis* can also infect cattle. These infections will occur principally by respiratory route but there have been reports of farmers with genitourinary TB due to *M. bovis* infecting cattle by urinating in cowsheds (Grange, 2001). This may constitute a risk in developing countries, where people have no sanitary facilities and often urinate on pastures (Ayele *et al.*, 2004).

Transmission of BTB can be influenced by several factors, with a three-level classification: individual, herd and region/country level (Figure 4). The situation in developed and developing countries might be different in terms of risk of BTB, i.e., farming practices, presence of wildlife, stocking density, pasturing systems and contact between animals (Humblet *et al.*, 2009). Thus, in developing countries the main risk factors are related to the intensification of the farms, and the lack of measures to control the disease.

In general, contamination of feed and pasture is not an important source of transmission of *M. bovis* in cattle, because survival times of infective doses of organisms on fomites are relatively short under real conditions and animals are not commonly exposed to a dose high enough to be infective by the oral route (Morris *et al.*, 1994). In natural infections occurring via the respiratory tract, *M. bovis* can be detected in nasal mucus from around 80 – 100 days after infection (Neill *et al.*, 1994).



**Figure 4. Main bovine tuberculosis risk factors classified into animal, herd and region/country levels (Humblett *et al.*, 2009).**

### 1.4.3 Prevalence and transmission of BTB among non-bovine species

*M. bovis* affects mainly cattle. However, man, domestic animals and wildlife can also be infected (Acha & Szyfres, 2001). Domestic animal species in close contact with infected cattle can acquire the disease mainly by the digestive route, i.e., sheep, llamas, goats, pigs, cats and dogs, whereas horses are rarely infected (Phillips *et al.*, 2003). These domestic animals are considered to be spillover hosts. BTB is usually maintained in cattle populations, but a few other species can become reservoir hosts. Most species are considered to be

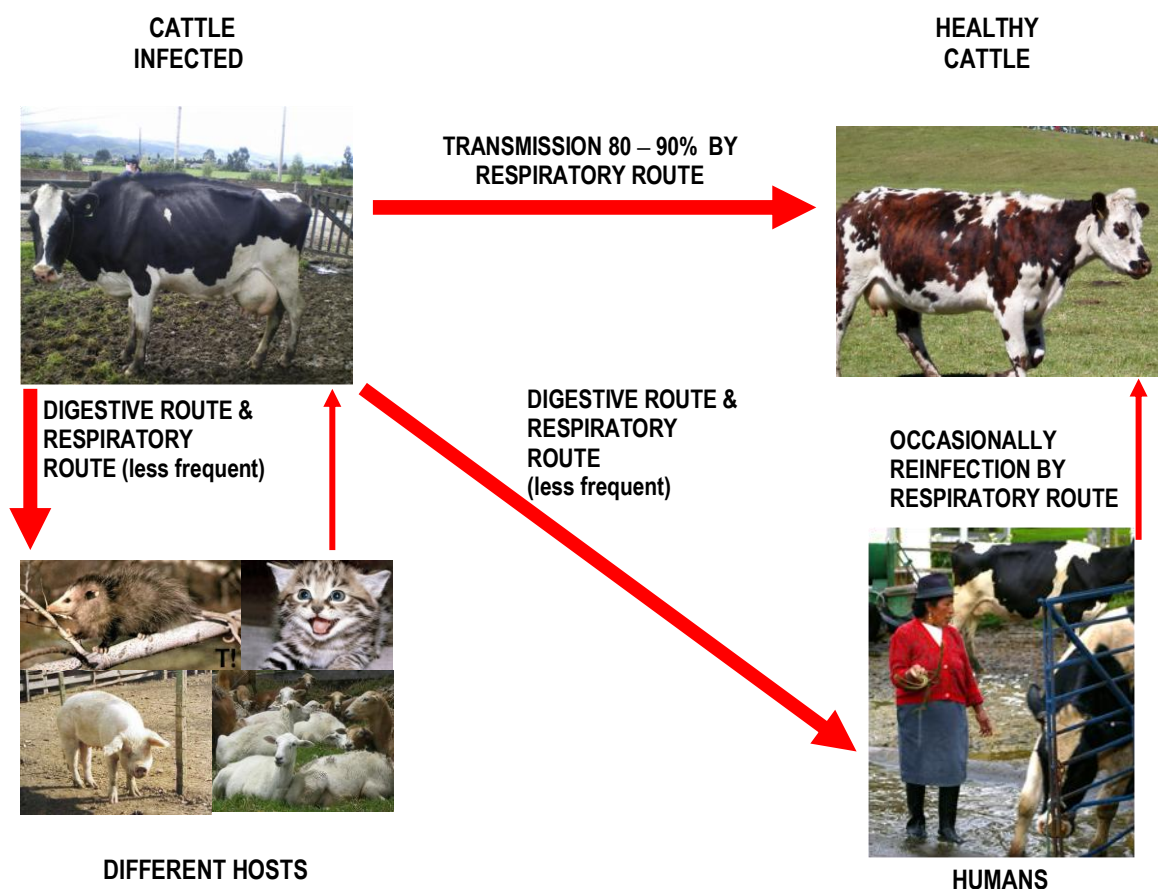
spillover hosts. Populations of spillover hosts do not maintain *M. bovis* indefinitely in the absence of maintenance hosts, but may transmit the infection between their members or to other species for a time. Some spillover hosts can become maintenance hosts if their population density is high (Morris *et al.*, 1994; CFSPH, 2007).

In sheep, experimental studies conducted by Chaussé (1913) showed that the effective dose to cause the disease by respiratory route is no more than 5 and probably as few as one bacillus would produce a lesion in the lung, whereas 13 million bacilli would not always infect sheep by the oral route.

In pigs, the occurrence of BTB can increase with age. In this species, the main route of transmission is the digestive tract; mainly by consumption of milk or dairy products, kitchen and abattoir scraps, and excreta from tuberculous cattle (Acha & Szyfres, 2001). Although transmission between swines can occur, it is considered to be epidemiologically no relevant (Morris *et al.*, 1994). In farms with a high prevalence of BTB, up to 50% of the cats may be infected (CFSPH, 2007).

*M. bovis* infection in wildlife and feral species is a potential source of infection for livestock and a threat to protected and endangered species (Aranaz *et al.*, 2004). In some countries, TB in cattle could be a readily controllable disease in absence of some wildlife reservoir (Morris *et al.*, 1994). In 22% of the countries, BTB has been detected in wildlife species, according to the report from the Office International des Epizooties (OIE) (Livingstone, 2000). In Europe and North America, some wild animals can frequently be affected. Known maintenance hosts are red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), wild boar (*Sus scrofa*) and iberian lynx (*Lynx pardina*) in Spain, badger (*Meles meles*) in the

UK and Ireland, brush-tailed possum (*Trichosurus vulpecula*) and ferret (*Mustela furo*) in New Zealand, deer (*Axis axis*) in the United States, bison (*Bison bison*) in Canada and ungulates in other countries. In Africa, infections have been reported in lion (*Panthera leo*), cheetah (*Acynonyx jubatus*), and other wildlife species (Aranaz *et al.*, 2004; Whipple & Palmer, 2000). The maintenance wildlife hosts are characterized by their ability to produce temporally continuous but spatially very patchy distribution of infection in their own population. The route of transmission varies between species and can be observed in Figure 5. The vast majority of spread within wildlife is by the airborne route (Morris *et al.*, 1994). Also among badgers, transmission appears to be mainly by respiratory route, and often it occurs as a result of fighting between males (Morris *et al.*, 1994). Cattle may be infected by badgers through direct or indirect (namely urine, faeces and pus) transmission.



**Figure 5. Routes of bovine tuberculosis transmission**

#### **1.4.4 Prevalence and transmission of BTB among humans**

In humans, TB induced by *M. bovis* or *M. tuberculosis* is not clinically different (Cosivi *et al.*, 1998). In the progressive stage of disease, the following symptoms can become evident: subfebrile temperatures, night sweats, lymphadenopathy, cough, fatigue, loss of appetite and erythema on the extremities (Krauss *et al.*, 2003). In immunodepressed patients, pneumonia lymphadenopathy or disseminated disease can be observed (Acha & Szyfres, 2001).

The main risk factors involved in BTB infections are consumption of contaminated milk or dairy products, and close physical contact between humans and potentially infected animals (Acha & Szyfres, 2001). People who work in close contact with potentially infected animals, such as the dairy farm and abattoir workers and also veterinarians run a high risk (Krauss *et al.*, 2003). Although consumption of meat from tuberculous cattle presents a theoretical risk, the main hazard comes mainly from cooking and preparation methods (Shakespeare, 2002).

In countries where BTB is not controlled, most of the human cases occur in young people as a result of unpasteurized milk consumption, which is an important source of TB due to *M. bovis* for humans. In these patients, cervical lymphadenitis, intestinal and skin lesions are frequently observed (Gil & Samartino, 2001). The respiratory disease in human population at risk may be acquired by inhaling droplet nuclei produced during cough spray from infected cattle (Grange, 2001).

Many laboratories do not distinguish between *M. bovis* and *M. tuberculosis* infections, and the documented cases on zoonotic TB are scarce, and even more the cases of TB due to *M. bovis* as a result of human to human transmission leading to disease occurs very rarely. Nevertheless, in 1994 this kind of infection was reported in The Netherlands (van Soolingen



*et al.*, 1994). Although in general it would be very difficult to prove the association because usually many years elapse between infection and development of bacteriologically positive disease (Grange, 2001).

In Latin America and the Caribbean, epidemiological data related to TB are scarce, mainly because the diagnosis of human TB is generally limited to sputum smear examination by Zielh Neelsen stain, which cannot differentiate among the various species of mycobacteria. Furthermore, samples are cultured mainly on Löwenstein-Jensen medium containing glycerol, which inhibits growth of *M. bovis* (Zumarraga *et al.*, 1999; Abalos & Retamal, 2004).

According to de Kantor & Ritacco (2006), 21 million people in 15 Latin American and Caribbean countries are living in close contact with cattle, including meat processing and veterinary services workers, cattle breeders and farmers. In addition, relatively high incidences of human TB cases (30 to 100 per 100000 inhabitants) are reported in those countries. It has been estimated that an annual incidence of 7000 new TB cases due to *M. bovis* occurs in this region.

In Argentina, *M. bovis* was identified in 2.3% of 4243 isolates from TB patients. Most zoonotic TB cases in the region were associated with infection via the respiratory tract, and 65% of the cases occurred among persons at risk due to close contact with animals. Milk pasteurisation and veterinary control in slaughterhouses have improved significantly here during the last decade. These measures are effective to decrease the risk of oral transmission but slaughterhouse and farm workers remain at risk of aerosol-borne pulmonary disease (Torres, 2009; de Kantor & Ritacco, 2006).

In Mexico, it has been demonstrated that *M. bovis* plays an important role in human TB (Zendejas-Martinez *et al.*, 2008). Human infection occurred mainly by the oral route, specifically by the consumption of cheese. *M. bovis* has been isolated in cheese, representing a high risk for public health. It has been calculated that 30 to 40% of the milk used to make cheese and other dairy products is sold raw (unpasteurized) (Perez-Guerrero *et al.*, 2008). In San Diego-USA, close to the border with Mexico, approximately 7% of the culture-positive TB patients had disease caused by *M. bovis* during the last decade; 90% of this group had Hispanic origin (LoBue, 2006).

In Ecuador, the real occurrence of human TB from animal origin is most probably underdiagnosed because the identification of the etiological agent is not performed as a routine procedure. Diagnosis of human TB is mostly restricted to direct microscopy, and Stonebrink medium - needed to grow *M. bovis* - is only used rarely. According to the National Reference Laboratory of infectious diseases, only two cases of TB due to *M. bovis* have been confirmed by culture (de Kantor *et al.*, 2008). In 2007, a tuberculin skin testing survey conducted in 157 farm and slaughterhouse workers (Benítez-Capistros, 2007), showed 29% of positive reactors with a significant association among the positive-TST and the habit of drinking raw milk ( $p < 0.00$ ), as well as with the consumption of cheese ( $p = 0.003$ ).

## **1.5    *Diagnosis***

### **1.5.1   *Ante-mortem (in vivo) diagnosis***

#### **1.5.1.1   *Clinical diagnosis***

Diagnosis of BTB based on clinical signs alone is not possible, because most of cattle do not present symptoms before an advanced stage of the disease, and these clinical signs are not

specific. The severity of the disease varies with the dose of infectious organisms and individual immunity. In the late stage, some signs may be suggestive such as gradual loss of condition, emaciation, low-grade fluctuating fever, weakness and inappetence. Occasionally, when the retropharyngeal nodes are enlarged, cattle present dysphagia. In case of advanced pulmonary disease, a chronic soft cough is present, which is worse in the morning, during cold weather or exercise, and may have dyspnea or tachypnea. In the terminal stages (Figure 6), animals may become extremely emaciated and develop acute respiratory distress (OIE, 2006; Neill *et al.*, 1994).



**Figure 6. BTB-infected cow from a dairy Ecuadorian herd** (Photo: F. Proaño-Pérez)

BTB can also affect the mammary glands and often produce palpable swellings of the upper part of the udder and supra-mammary nodes. Greatly enlarged lymph nodes can also obstruct blood vessels, airways, or the digestive tract. When the digestive tract is involved, intermittent diarrhoea and constipation may be seen (Gallagher & Jenkins, 1998). The differential

diagnosis of BTB includes contagious bovine pleuropneumonia, *Pasteurella* or *Corynebacterium pyogenes* pneumonia, and aspiration pneumonia.

In maintenance hosts other than cattle, the severity of the disease varies with the species (Neill *et al.*, 1994). Brush-tailed possums are highly susceptible to BTB, and usually it causes a fulminating pulmonary disease. In the final stage, they become disoriented, can not climb, and sometimes can be seen wandering about in daylight. In badgers, on the contrary, BTB does not frequently develop visible lesions and animals can survive for many years with the bacilli. In symptomatic badgers, BTB is primarily a respiratory disease (Morris *et al.*, 1994).

#### **1.5.1.2 The tuberculin test**

In human and veterinary medicine, intradermal tuberculinization or the tuberculin test is the oldest and the most widely used *in vivo* diagnostic test. Tuberculin tests have been used for the diagnosis of BTB in cattle for more than 100 years, and it is still widely used in the field due to its ease of application on a large scale in livestock and low cost. Eventhough its limitations have been recognized for many years, the tuberculinization has been the key procedure in national test and slaughter programmes for BTB and its use has facilitated the eradication of the disease in many regions of the world. Furthermore, it has provided most of the available data on tuberculosis (Acha & Szyfres, 2001; Mongham *et al.*, 1994).

Consequently, rigorous application of tuberculin testing and culling reacting cattle resulted in the control of *M. bovis* infections; in the United States, it allowed to control BTB in domestic livestock but without complete eradication (Whipple & Palmer, 2000).

This diagnostic method depends upon the cell-mediated immunological response of the host during the development of the disease (Adams, 2001). Wax D and various tuberculoproteins induce a delayed hypersensitivity reaction detected in the tuberculin test (Quinn *et al.*, 1999). Primary TB can be diagnosed only by the development of a positive skin test (Krauss *et al.*, 2003). The standard procedure used for routine diagnosis of BTB by tuberculin test consists of reading the skin reaction to an intradermally injected purified protein derivative (PPD) (Shakespeare, 2002). Since 1975, PPD production switched to use *M. bovis* AN5, a strain originally isolated in England and used nowadays worldwide for bovine PPD production. The genome analysis showed that *M. bovis* AN5 has not suffered extensive gene deletion events and it can be used for the detection of infection by *M. bovis* strains that are currently prevalent (Hewinson *et al.*, 2006).

Sensitivity of tuberculin tests has been reported in ranges from 77 to 95%, and specificity from 96 to 99% (Mongham *et al.*, 1994). The cutoff points can influence the sensitivity and specificity of the skin tests i.e, if the cut-off is lowered, more infected animals are detected but more non-infected animals are identified as false positives as well (Mongham *et al.*, 1994; Demelash *et al.*, 2009).

The tuberculin test depends on several factors including high-quality reagents as well as the immunological status of the animal. False-positive reactions are partly explained by allergies to the simultaneous use of avian tuberculin, hypersensitivity to NTM, *Corynebacterium*, *Fasciola hepatica*, and *Nocardia* species (Sreevatsan *et al.*, 2000). False-negative responses occur in animals in the early or in the late stages of the disease if anergy develops due to excess of antigen or poor immune responses. Nonspecific factors such as malnutrition, stress or recent calved, are alternative causes of anergy. Desensitization may be observed if time

intervals between tuberculinizations are too short or if the procedure is not respected (injection of insufficient tuberculin, use of tuberculins of reduced potency). The variability among observers is also a source of errors (Biberstein & Hirsh, 1999). BCG vaccination sensitises animals to antigens present in the bovine PPD and interferes with the tuberculin skin test diagnosis in BCG vaccinated animals (Buddle *et al.*, 1999).

#### **1.5.1.2.1      *Simple intradermal tuberculin test (SITT)***

The SITT is a screening skin test mostly used in animals to identify possible reactors to BTB, which is based on the dermal reaction obtained in animals that have had a previous contact with the bacillus. In cattle, the skin areas used to perform this test are the skin of the neck or in the caudal, vulvar or anal fold (Figure 7). The selected area needs to be cleaned, and the tickness of the skin measured with callipers. This procedure is carried out before the injection and when reading the result after 72 hours (+/- 6 hours). A dose of 0.1 mL of bovine PPD needs to be injected intradermally, and should not be lower than 2000 International Units (IU). The interpretation of the SITT considers a positive reaction if a swelling of 5 mm or more at the side of the injection developed in 72 hours. This suggests a past or present infection. An inconclusive reaction is considered if the increase in skin-fold thickness is between 3 and 4mm, whereas for a negative reaction, the increase is less than 3mm without clinical signs. Animals with positive or inconclusive reactions may be subjected to a further SITT or confirmatory skin test after a period of minimum 42 days. In some areas, this procedure is conducted after only 60 days (Mongham *et al.*, 1994; OIE, 2006). It should be kept in mind that the skin of the neck is much more sensitive than the skin of the caudal fold. For this reason, the former site is always used in a second test to discard the false positives by the first tuberculinization.

#### **1.5.1.2.2      *Comparative intradermal tuberculin test (CITT)***

The CITT is a common skin test used mainly in suspected and positive reactors identified by the SITT to confirm BTB. It is used mainly to differentiate between animals infected with *M. bovis* and those sensitised to tuberculin due to exposure to NTM or related genus. The CITT is based on the simultaneous use of bovine and avian PPDs (PPD-B & PPD-A). This procedure is applied in the middle third of the neck, in this area two different spots need to be shaved and cleaned at least 12 cm apart (Figure 8). The thickness of the skin is measured with callipers before the injection of the antigens: 0.1 mL (25 000 IU/mL) of PPD-A and 0.1 mL (20 000 IU/mL) of PPD-B. 72 hours after PPD injections, skin thicknesses are measured again to identify positive reactors.

A positive reaction to CITT is defined as a relative increase in skin thickness at the injection site for PPD-bovine of at least 4 mm greater than the increase in skin thickness at the injection site for PPD-avian. The reaction is inconclusive if no clinical signs are observed and if the increase of the skin thickness is more than 2 mm and less than 4 mm. A negative reaction is considered if only limited swelling is developed with an increase of no more than 2 mm and without clinical signs. (OIE, 2008; Grooms & Molesworth, 2000).



**Figure 7. Simple intradermal tuberculin test (SITT)**  
(Photo: F. Proaño-Pérez)



**Figure 8. Comparative intradermal tuberculin test (CITT)**  
(Photo: F. Proaño-Pérez)



## **1.5.2 Post-mortem inspection**

### **1.5.2.1 Necropsy procedure**

The necropsy procedure performed after slaughtering of animals allows for the detection of gross lesions suggestive of BTB, even in apparently healthy cattle. Veterinary inspection is established as a routine procedure in most of the slaughterhouses in developing countries. In the later stages of an eradication campaign and when the prevalence of BTB is low, the detection of infected animals is mainly restricted to the routine slaughterhouse inspection (Corner, 1994).

When detailed necropsy is conducted on reactor cattle, different tissues need to be sampled (Table 2). In order to identify gross lesions compatible with BTB, careful examination needs to be carried out. Although this method is used to identify infected animals, it can be affected by the technique used and the anatomical sites examined (Corner, 1994). According to Demelash *et al.* (2009), the sensitivity of the necropsy has been calculated in 95%, when as few as 6 pairs of thoracic lymph nodes, lungs, and mesenteric lymph nodes are inspected. The sensitivity of the necropsy procedure depends also largely on the time and diligence of the inspectors during the examination of the carcasses (Corner, 1994).

Necropsy can fail to detect infected animals in an early stage of infection or if observation and sampling are not complete. In addition, not all *M. bovis*-infected cattle develop visible gross lesions (Whipple *et al.*, 1996). Lesions due to an infection with non-tuberculous mycobacteria (NTM) can be easily mistaken with BTB lesions (Oloya *et al.*, 2007). Therefore, it is important to confirm the presence of *M. bovis* by the use of available laboratory methods.

**Table 2. Tissues to be examined for the presence of macroscopic lesions in cattle reacting to a tuberculin test.**

<b>Regions</b>	<b>Tissues</b>
Head	mandibular lymph nodes* parotid lymph nodes* medial retropharyngeal lymph nodes* lateral retropharyngeal (atlantal) lymph nodes* Tonsils
Thorax	mediastinal lymph nodes** tracheobronchial lymph nodes* bronchial lymph node*** Lungs
Abdomen	Liver hepatic lymph node Spleen mesenteric lymph nodes (along the entire length of the gastro-intestinal tract) Kidneys
Carcasse	caudal cervical (prescapular) lymph nodes* subiliac (prefemoral) lymph nodes* internal iliac lymph nodes* medial iliac lymph nodes* lateral iliac lymph nodes* gluteal (ischiatric) lymph nodes* sacral lymph nodes* superficial inguinal (supramammary or scrotal) lymph nodes* udder or scrotal contents and seminal vesicles

\* = left and right

\*\* = anterior and posterior

\*\*\* = cranial and medial

(From Corner *et al.*, 1990)

### 1.5.2.2 Macroscopic lesions

BTB is characterized by the development of granulomas or tubercles where the bacilli are localized. When *M. bovis* is transmitted in a new host, a primary lesion or focus of infection is established, which depends on the interaction between host and pathogen. This primary lesion, together with the lesion in the regional lymph node is called the “primary complex” (Neill *et al.*, 1994). The granulomas are usually yellowish and either caseous, caseo-calcareous or calcified, and often encapsulated (Figure 9). A focal caseous necrotic centre or mineralization can be observed commonly. Non-tuberculous granulomas in which the purulent content is replaced by granulation tissue, may resemble to tuberculous granulomas (OIE, 2006; Whipple *et al.*, 1996).



**Figure 9. Hepatic lymph node from a tuberculous cow**

(Photo: F. Proaño-Pérez)



**Figure 10. Mediastinal lymph node from a tuberculous cow, showing a yellowish caseous granuloma (Photo: F. Proaño-Pérez)**



**Figure 11. Lung from a tuberculous cow showing calcified granulomas (Photo: F. Proaño-Pérez)**

BTB infection in cattle mainly occurs by inhalation of *M. bovis* in droplet nuclei. Thus, the primary complex is usually found in the lungs and associated lymph nodes. In cattle, granulomas are located mainly in the lymph nodes from head and thorax; however, they are also common in the lungs, spleen, liver, and the surfaces of body cavities (Figures 10 and 11). In disseminated cases, multiple small granulomas may be found in numerous organs (Cousins *et al.*, 2004). Lymph node lesions may be absent in cases of chronic tuberculosis pneumonia.

According to Neill *et al.* (1994), 57% of cattle present lesions confined to the bronchial and/or mediastinal lymph nodes, 23% in the head only (retropharyngeal and submaxillary lymph nodes) and a lower occurrence of 3.2% in mesenteric lymph nodes. However, it is important to consider that the distribution of lesions can vary depending on several factors, i.e., route of transmission, infecting dose, incubation period before examination, virulence, individual immune response, age or breed (Corner, 1994; Neill *et al.*, 1994; Whipple *et al.*, 1996). In some species such as deer, the lesions tend to resemble abscesses rather than typical tubercles. Some tubercles are small enough to be missed by the naked eye, unless the tissue is sectioned (CFSPH, 2007).

### **1.5.3 Laboratory diagnostic tools**

*M. bovis* can be identified by various laboratory methods. Samples collected from suspicious animals are commonly used to confirm the presence of *M. bovis* by applying microscopy, histopathology, *in vitro* culture, PCR, and blood testing. They must be treated as risk/hazard group III to prevent human infections during their processing (OIE, 2006).

### 1.5.3.1 Microscopy

Tubercle bacilli may be demonstrated in secretions and biopsies from animals and humans by microscopy. It remains as the most used method for confirming TB in developing countries with suitable cost and rapid results. The acid fastness of the bacilli is normally demonstrated with the Ziehl-Neelsen stain, a fast and easy procedure. However, the disadvantage of microscopy is the lack of specificity and sensitivity. It can not distinguish between the various species of the family Mycobacteriaceae (Vitale *et al.*, 1998; Zumarraga *et al.*, 1999), and it requires a large numbers of organisms in the sample to yield a positive result. More than  $5 \times 10^4$  mycobacteria/mL sample must be present to make detection reliable. The numbers of *M. bovis* bacilli is often low in bovine specimens (Quinn *et al.*, 1999; Boddingtonhaus *et al.*, 1998). According to Cambanis *et al.* (2007), spending at least 10 minutes per slide in the examination can significantly increase case detection.

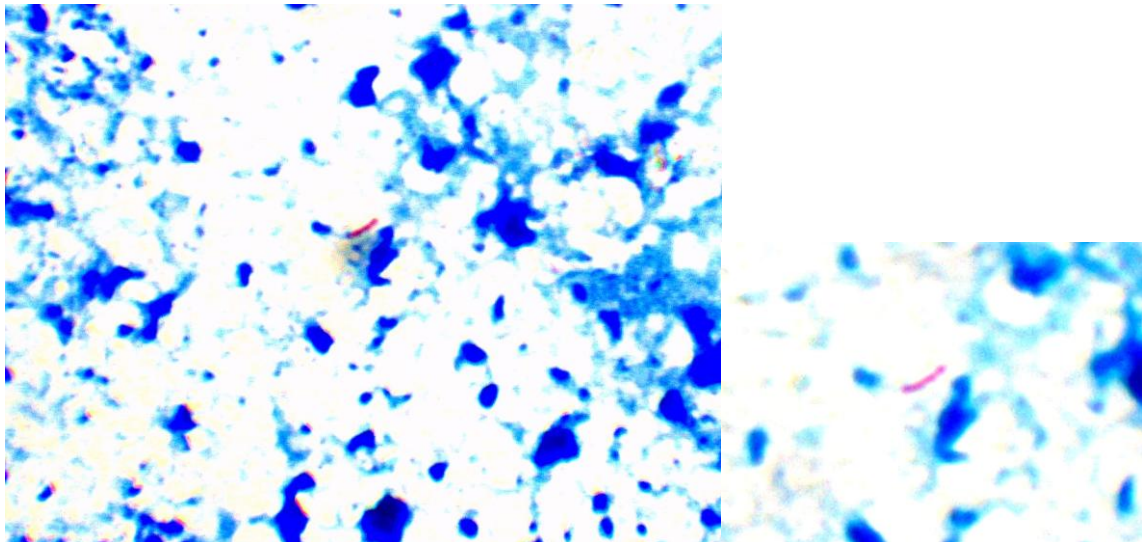
*M. bovis* bacilli appear as slender, often beaded, red-staining against a blue background (if methylene blue is the counterstain) (Figure 12). They are generally shorter than *M. tuberculosis* bacilli and can be found forming clumps, but they form cords less frequently than *M. tuberculosis* (Cardoso *et al.*, 2004).

### 1.5.3.2 Histopathology

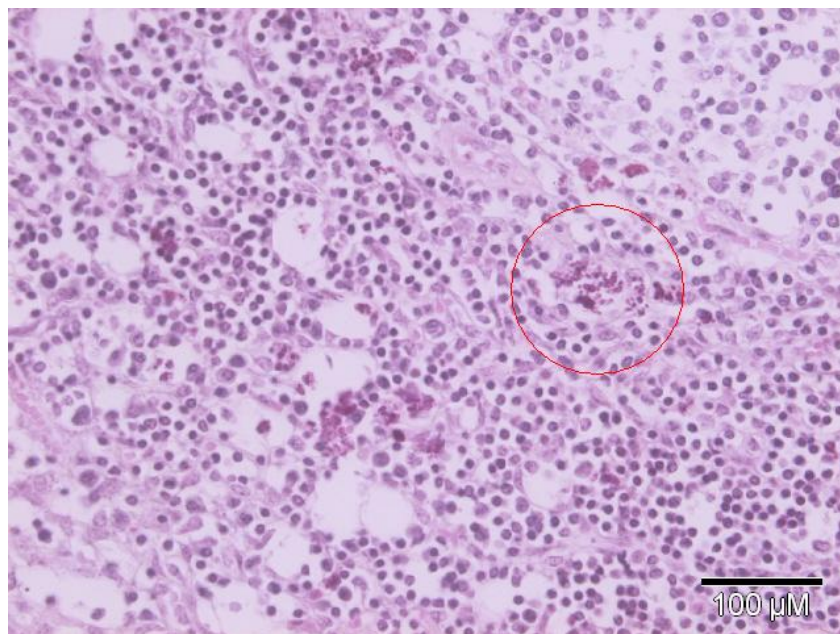
In high prevalence areas, the histological diagnosis is a useful approach but it cannot be relied upon in all situations (Corner, 1994). Histopathologic examination is a reliable tool for rapid diagnosis compared with culture, and used in countries where active BTB eradication programs allow the prompt identification and elimination of reactor cattle. In addition, this



method permits identification of typical mycobacterial lesions and its differentiation from other causes (Varello *et al.*, 2008).



**Figure 12.** *Mycobacterium bovis* bacilli in a smear stained with Ziehl Neelsen (magnification 1000x) (Photo: F. Proaño-Pérez)



**Figure 13.** Macrophage in a bronchial lymph node specimen stained with Ziehl Neelsen (Photo: D. Desmecht & F. Proaño-Pérez)

The histological picture shows typically tuberculous with central caseation necrosis and calcification, with or without mineralization. At the periphery of the lesion, unaltered macrophages are mixed with lymphocytes. Fibrocytes and fibrous layer appear to form a tubercle (Figure 13). Within the foci, macrophages often assume distinctive appearance (epithelioid cells) and giant cells or the Langhan's type (Neill *et al.*, 1994). Epithelioid cells appear among the macrophages. They have oblong vesicular nuclei and pale, poorly delineated cytoplasm; they often become the predominant cells. The Langhan's giant cells are probably a macrophage fusion product, which have a large amount of pale cytoplasm and peripherally, many vesicular nuclei. Both cells are probably macrophages derivatives, which could not be effective phagocytes (Biberstein & Hirsh, 1999).

#### **1.5.3.3 *In vitro* culture**

*In vitro* culture is used routinely to confirm the presence of *M. bovis* in post-mortem specimens. Especially when the disease prevalence is low, as during the latter stages of an eradication campaign, the need for a definitive diagnosis is strategic. However, the success of its primary isolation depends on the decontamination procedure and incubation conditions (Corner, 1994). A common decontamination procedure for the isolation of *M. bovis* from biopsies is the inverted Petroff method (Portaels *et al.*, 2001). Homogenised tissues are mixed with 1N HCl for 20 min, followed by a neutralisation with 1N NaOH and concentration of the bacilli by centrifugation.

Culturing the bacillus is an extremely slow (generation times ranging from 2-20 hours) and laborious procedure, requiring 6 to 8 weeks of growth at 34 – 38°C. Approximately 10 to 100 viable organisms are required to yield a positive culture. This high number of bacilli usually



occurs only in advanced stages of the disease. For *in vitro* culture of *M. bovis*, Stonebrink or Coletsos medium are widely used. They are adapted Lowenstein-Jensen media without glycerol and with addition of sodium pyruvate (Figures 14 and 15). The presence of glycerol inhibits the growth of *M. bovis*. Biochemical and enzymatic tests can be applied for the *in vitro* identification (Table 3). *M. bovis* is characterized by rough colony morphology, absence of niacin production, nitrate reduction, catalase and pyrazinamidase negative activity, presence of urease activity, and susceptibility to thiophene-2-carboxylic acid hydrazide and *p*-animo-salicylic acid (Biberstein & Hirsh, 1999; Gallagher & Jenkins, 1998).

**Table 3. *In vitro* tests for some significant mycobacteria**

species	Runyon group	Growth within 7 days	Inhibition by glycerol	Colonial morphology *	Pigmentation **	Niacin production	Tolerance of 5% NaCl	Deamination of PZN	Nitrate reduction	Urease production
<i>M. bovis</i>		neg	pos	S/R	n	neg	neg	neg	neg	v
<i>M. tuberculosis</i>		neg	neg	R	n	pos	neg	pos	pos	v
<i>M. avium intracellulare</i>	III	neg	neg	S/R	n	neg	neg	pos	neg	neg
<i>M. ulcerans</i>	III	neg	neg	R	n	neg	neg	neg	neg	v
<i>M. simiae</i>	I	neg	neg	S	p	v	neg	pos	neg	pos
<i>M. kansasii</i>	I	neg	neg	SR	p	neg	neg	neg	pos	v
<i>M. marinum</i>	I	neg	neg	S/SR	p	-(+)	neg	pos	neg	pos
<i>M. scrofulaceum</i>	II	neg	neg	S	s	neg	neg	v	neg	v
<i>M. xenopi</i>	III	neg	neg	S	n(s)	neg	neg	v	neg	neg
<i>M. chelonae</i>	IV	pos	neg	S/R	n	v	+(-)	pos	neg	pos
<i>M. fortuitum</i>	IV	pos	neg	S/R	n	v	pos	pos	pos	pos

\* R = rough, S = smooth, and SR = intermediate

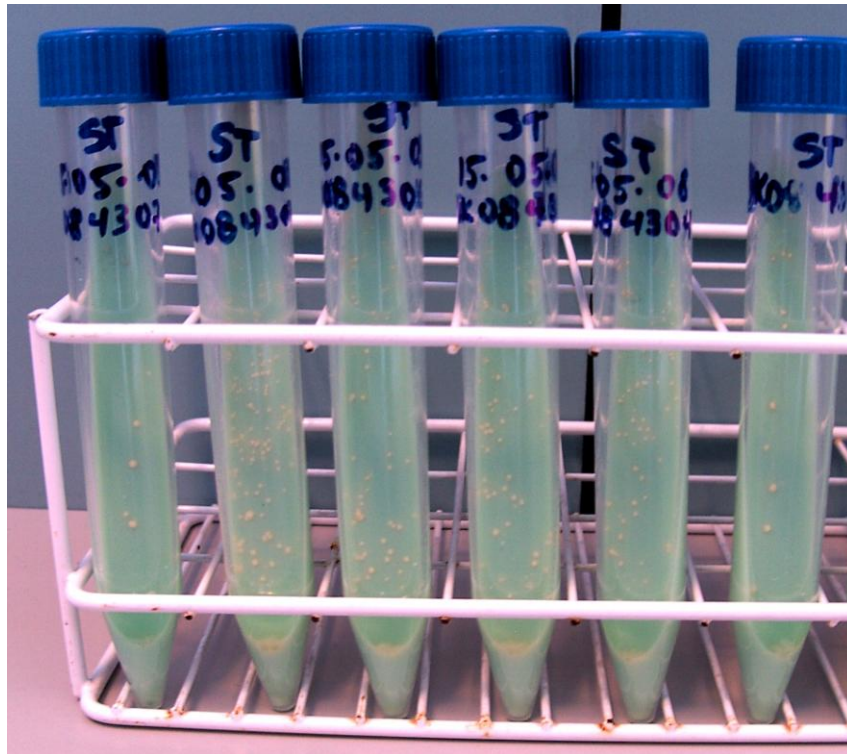
\*\* p = photochromogenic (pigment produced only if culture is exposed to light)

s = scotochromogenic (pigment produced in the light and in the dark)

n = non-chromogenic (no pigment produced)

v = variable reactions, -(-) = majority negative, +(-) = majority positive

(From Quinn *et al.*, 1999)



**Figure 14. Colonies of *M. bovis* on Stonebrink medium after 8 weeks of incubation**  
(Photo: F. Proaño-Pérez)



**Figure 15. Colonies of *M. bovis* on Stonebrink medium after 24 weeks of incubation**  
(Photo: F. Proaño-Pérez)

Corner & Nicolacopoulos (1988) recommended the use of Stonebrink, Middlebrook 7H11 or tuberculosis bovine blood agar as a media of choice for the primary isolation of *M. bovis*. In addition, liquid media with a radiometric detection technique can be used i.e., BACTEC® system (Middlebrook *et al.*, 1977). This method is based on a radiolabelled palmitic acid on liquid medium, which allows a rapid detection of growing bacilli that produce radiolabeled CO<sub>2</sub>. The disadvantage of the BACTEC culture is that it can be performed only in a high level laboratory, that it is labor intensive and poses a risk of exposure to radiation. Nowadays, a novel culture method has been evaluated as a suitable alternative for the replacement of the BACTEC 460 radiometric system, the BACTEC Mycobacteria Growth Indicator Tube (MGIT) system, which rapid, and based on a non-radiometric fluorescent detection system (Hines *et al.*, 2006). However, again the automated version requires expensive equipment and media.

#### **1.5.3.4 Immunological tests**

Blood-based immunological assays can be used to diagnose BTB, especially in zoo animals and wildlife. The available tests are the lymphocyte proliferation and gamma-interferon assays, both measuring cellular immunity. The lymphocyte proliferation test is not commonly used in cattle, but may be useful in wildlife and zoo animals, as they must be captured only once, rather than twice for the tuberculin test. The enzyme-linked immunosorbent assay (ELISA) may complement tests of cellular immunity in anergic cattle. There is no universally accepted diagnostic blood test for BTB in cattle or other animals (OIE, 2006; Thoen & Ebel, 2006).

Sensitivity and specificity of ELISA to diagnose TB depends on the antigen used; in general this test has a high specificity but its sensibility is poor (Mahavir & Espitia, 2007).

All these methods present technical restrictions, which in general are expensive and the availability of laboratories and trained staff is a challenge. In addition, these tests may be influenced by factors such as age, immunological state and exposure to NTM strains (Wood & Rothel, 1994; Mahavir & Espitia, 2007).

#### ***1.5.3.4.1      Gamma interferon assay***

Gamma-interferon ( $\gamma$ -IFN) is a cytokine predominantly released by T lymphocytes after antigenic stimulation, which has an important role in the immune response to tuberculous mycobacteria as the major macrophage activation factor.

The test is mainly used in cattle and it is based on the liberation of the  $\gamma$ -IFN from the sensitised lymphocytes, the amount of  $\gamma$ -IFN in the plasma is quantified by a sandwich ELISA (Krauss *et al.*, 2003; de la Rúa *et al.*, 2006). It detects the immuno responses to the ESAT-6 protein from tuberculosis-infected and environmentally-sensitized herds that have been in contact with the bacillus (Pollock & Andersen, 1997).

The  $\gamma$ -IFN assay results correlate well with responses obtained on a tuberculin test. This procedure allows allay concerns about the specificity of the tuberculin skin tests. The problem of false-positive reactions due to the cross-reactive in the tuberculin skin tests can be overcome by using a  $\gamma$ -IFN assay, when both methods can be applied and it is possible to compare results (Wood & Rothel, 1994).

Blood samples for the  $\gamma$ -IFN test must be transported to the laboratory promptly, as this test must be started within 24 to 30 hours after blood collection. However, the main disadvantage is the high cost (i.e., approx. 50 USD) of the  $\gamma$ -IFN limiting its use especially in developing countries (Thoen & Ebel, 2006).

### **1.5.3.5 Polymerase chain reaction (PCR)**

Differentiation of the causative *Mycobacterium* species usually requires *in vitro* growth of the organism, which is time consuming. Alternatively, PCR has been developed with considerable success (Shakespeare, 2002). PCR has been successfully applied to identify *M. tuberculosis*-complex DNA directly in samples from animals and humans like sputum, blood, milk, nasal swabs and tissues. The advantage of this method versus culturing is its rapidity of detection and ability to detect non-viable bacilli. PCR significantly reduces the time to confirm diagnosis in suspected cases (Boddinghaus *et al.*, 1990). However, its relative high cost is considered to be prohibitive for extensive routine diagnosis (i.e., approx 40 \$USD). Therefore, PCR is mostly restricted for research purposes in developing countries. The detection limit reported by PCR-based methods can vary between 1 and 100 bacilli present in the sample (Zumarraga *et al.*, 2005).

The primers most commonly used in *M. bovis* PCR-based assays target IS6110, an insertion sequence (IS) that usually has more than 10 copies in the genome of *M. tuberculosis*, while *M. bovis* contains in general a single copy (Thierry *et al.*, 1990). Boddinghaus and others (1990) have developed a sensitive and rapid technique based on the amplification of nucleic acid sequences from 16S rRNA gene, a gene present in all prokaryotes. The rRNA is an essential constituent of bacterial ribosomes, with highly conserved sequences but also a

considerable amount of variability. It is present in large copy numbers ( $10^3$  to  $10^4$  molecules per cell) (Bates, 1979). This technique is performed by using specific primers directed to the 16S rRNA gene and allows identifying organisms belonging to the genus *Mycobacterium* and members of the *M. tuberculosis* complex.

The 500-bp genomic fragment is conserved among *M. bovis* strains and is used on PCR-based assay to identify BTB (Rodriguez *et al.*, 1999). This method is able to detect down to 10 fg DNA, which corresponds roughly to two bacilli and can be used directly on biological samples such as milk making this a good assay for the rapid and specific diagnosis of *M. bovis* and can be used as an epidemiological tool (Rodriguez *et al.*, 1995). The first studies on direct detection of *M. bovis* from bovine tissues by the use of PCR were reported in 1995 (Wards *et al.*, 1995; Liébana *et al.*, 1995).

The complex structure and impermeability of the cell walls of mycobacteria complicate the extraction of DNA and/or RNA (Boddinghaus *et al.*, 1990). In addition, the low numbers of AFB in tissues from BTB-infected cattle suggest that a sensitive extraction procedure is required for the PCR (Roring *et al.*, 2000). The sensitivity of the PCR can also be affected by the presence of inhibitors in the samples (Mangiapan *et al.*, 1996; Cardoso *et al.*, 2009). Besides, extraction of mycobacterial DNA from tissue specimens is more difficult than from sputa (Wards *et al.*, 1995). Therefore, the choice of DNA extraction method is a key factor determining the efficacy of diagnosis by PCR. Mangiapan and colleagues (1996) have developed a usefull extraction approach to improve the sensitivity of detection, based on the use of biotinylated primers that permit the specific capture of mycobacterial DNA prior to amplification.

According to Romero *et al.* (1999), this method can also be used in nasal mucus from live cattle, because 90% of the infection cases occur by respiratory route. This finding is supported by the observation that thoracic lesions are more frequently affected than those located in either the mesenteric region or mammary glands, more oftenly observed in disseminated infections. Nevertheless, collection nasal swabs could be difficult in field conditions if the veterinarians have no experience.

### **1.5.3.6 DNA fingerprinting techniques for identification and typing *M. bovis* strains**

#### ***1.5.3.6.1 Restriction fragment length polymorphism (RFLP)***

RFLP technique relies on digestion of genomic DNA followed by probing with a specific marker using Southern blotting techniques (Harris, 2006). IS6110-RFLP is the gold standard for typing of *M. tuberculosis*-complex strains. However, in strains with a low copy number of IS6110 such as *M. bovis*, this technique has some limitations (Hewinson *et al.*, 2006). The level of differentiation of *M. bovis* isolates obtained by IS6110-associated RFLP analysis depends on the origin of the stain (van Soolingen *et al.*, 1994). The copy number of IS6110 in clinical strains of *M. bovis* appears to be somewhat correlated with animal species, i.e., in cattle isolates generally carry one IS6110 copy, whereas strains harbouring multiple copies are more likely to origin from wildlife or zoo animals (Cousins *et al.*, 1998).

Another variation of RFLP for typing *M. tuberculosis*-complex identifies the polymorphic G/C-rich sequence (PGRS) loci, which are microsatellite regions in the mycobacterial genome consisting of multiple short tandem units of less than 10 bp (Harris, 2006). Isolates with the same spoligotype could be diferenciaded by RFLP analysis with the direct repeat (DR) and PGRS probes, the DNA rearrangements can be expected from variations in two

different repetitive DNA sequences. Consequently, a combination of two methods (spoligotyping, and typing with RFLP analysis with the DR and PGRS probes) would be necessary for the differentiation of *M. bovis* for a detailed epidemiological study (Zumarraga *et al.*, 1999).

#### **1.5.3.6.2 Spoligotyping**

Spacer oligonucleotide typing (Spoligotyping) is a useful molecular typing approach enabling to differentiate *M. bovis* strains, a distinction which is often difficult to make by traditional methods (Kamerbeek *et al.*, 1997). Spoligotyping is a simple, rapid and robust method for simultaneous detection and typing of *M. tuberculosis* complex, it is possible to carry out directly from tissue specimens (i.e., microscopy positive) without the need to culture the bacillus or to purify DNA (Roring *et al.*, 2000).

This method is based on PCR amplification of a high polymorphic DR locus within the genome of the *M. tuberculosis* complex, which contains DR sequences interspersed with variable spacer sequences. Each DR is 36 bp long and spacer length varies from 35 to 41 bp. The DR locus is a preferential site for insertion of a copy of the IS6110 element. The interpretation of the test result is based on the presence or absence of specific spacer region sequences between two DR regions, using labelled primers directed to this region followed by reversed line blot hybridization. The test format includes oligonucleotides allowing detection of 43 spacer sequences. The absence of spacers 3, 9, 16, and 39-43 is a characteristic of all *M. bovis* strains (Kamerbeek *et al.*, 1997).



Since it is relatively fast, it is in particular useful in low-incidence communities or when the study of an outbreak requires urgent differentiation (van Helden, 1999). A second-generation of the spoligotyping assay detects the presence of the 43 traditional spacers plus 51 new additional spacers oligonucleotides; however, it was detected a redundancy analysis and only 40 essential spacer oligonucleotides of the 94-spacer sequences were selected. This technique was performed in Netherlands and described by van der Zanden *et al.* (2002), which is used whenever extended discrimination is required (i.e., for low-copy-number IS6110 strains).

#### **1.5.3.6.3      *Variable number tandem repeats (VNTRs)***

A more recent PCR-based typing method based on 12 loci containing variable number tandem repeats (VNTRs)-genotyping of genetic elements named mycobacterial interspersed repetitive units (MIRUs). *M. tuberculosis* complex members have 41 different MIRUs randomly located throughout their genomes (Supply *et al.*, 2000; Roring *et al.*, 2002). Recently, an optimized set of 24 loci was defined, including a highly discriminatory subset of 15 loci for specific first-line epidemiological investigation (Supply *et al.*, 2006).

MIRU-VNTR includes a PCR amplification using primers specific for the flanking regions of the MIRUs, and studies the variability in number of repeats of these minisatelites in specific loci of the *M. tuberculosis* complex genome. VNTR-typing for *M. bovis* targets minimum six loci, whereas Spoligotyping only records variation at a single locus (Frothingham and Meeker-O'Connell, 1998; Supply *et al.*, 2000).

The VNTRs can be used for analysis of the global genetic diversity of *M. tuberculosis* complex strains at different levels of evolutionary, divergence opening the way for global

epidemiological surveillance of tuberculosis (Supply *et al.*, 2001) or ranging from pedigree to evolutionary distant phylogenetic relationships (Hewinson *et al.*, 2006). Certain MIRU-VNTR markers may be useful for larger population-based studies, whereas others may be more appropriate for outbreak analysis (Harris, 2006). This is the epidemiological typing method of choice for the near future and has 100% reproducibility and its use is being evaluated to accept it as a diagnostic tool (Kremer *et al.*, 2005).

## **1.6 Control of BTB**

The control of BTB is important not only because of its human health impact but also because of the resulting economic losses (OIE, 2006). In most countries, TB is a notifiable disease in humans and animals, and strict controls have to be exerted on affected herds. Control measures usually include early diagnosis with tuberculin test followed by slaughtering of infected animals.

The basic strategies required for control and elimination of BTB in Latin America are well known and well defined by the World Organization for Animal Health. Regular testing and removal of infected animals at intervals of less than a year, in combination with appropriate hygiene measures and restrictions to prevent introduction of infected animals could eliminate the infection from herds (Morris *et al.*, 1994). However, because of financial constraints, scarcity of trained professionals, lack of political will, as well as the underestimation of the importance of this zoonotic disease in both animals and public health sectors by national governments, these control measures are not applied or are applied inadequately in most developing countries (Cosivi *et al.*, 1998).

In Latin America, a “Plan of Action for the eradication of BTB in the Americas”, has been developed by the Pan American Health Organization (PAHO), in cooperation with the Pan American Institute for Food Protection and Zoonoses (INPAAZ) and the Food and Agriculture Organization (FAO). It aims to eradicate BTB from countries with more advanced national programs (Cosivi *et al.*, 1998).

In developed countries, control programs have been conducted with success and some countries are free of BTB. However, one of the greatest threats to any control program in domestic ruminants is wildlife reservoirs that cannot be controlled and can re-introduce the disease in livestock (Biet *et al.*, 2005). It is now recognised that wildlife reservoirs play a significant role in the persistence of *M. bovis* in cattle and make the eradication of the disease difficult (Corner, 2006), especially when domestic and wildlife species share the same habitat (Gallagher & Jenkins, 1998). The control programs are difficult if (1) BTB may persist in a wild population (maintenance hosts) without presence of infected cattle and if (2) there is a risk of contact between these wild populations and cattle. However, the only control measure available for wildlife hosts is removal of infected animals (Morris *et al.*, 1994).

BTB distribution might facilitate the work of the national control programme by identification of areas of higher or lower BTB transmission risk, complemented with environmental model that summarises conditions i.e., climate, topography, soils and land cover, under *M. bovis* is expected to be absent, versus potentially present. These environmental characteristics can be useful to study BTB transmission on spatial scales. In Mexico, the national BTB control campaign focused on involving farmers by means of certification of farm as BTB-free after 3 consecutive negative tuberculin test results; however, outbreaks in certified BTB free herds

raise the question if *M. bovis* can survive undetected and that environmental factors are involved (Zendejas-Martinez *et al.*, 2008).

*M. bovis* can enter into a herd from outside via cattle by two major routes, i.e., through purchase of infected cattle and from neighbouring farms by contiguous spread. According to Goodchild & Clifton-Hadley (2001), an adequate biosecurity program can reduce spread between herds, and improvements in management measures i.e., adequate ventilation, reduce of group sizes and hygienic measures can reduce the transmission within herds. Additionally, a complementary strategy need to be implemented to minimize the number of movements from infected to non-infected herds or areas, using the animal or herd identification to control the movements. However, if the infection persists under such circumstances, it is entirely due to management procedures allowing infected animals to remain in herds and to move between herds (Morris *et al.*, 1994; Phillips *et al.*, 2003).

To ensure the effectivity during the identification of bovine reactors, the production, standardization and quality control of purified protein derives is urgently required for use in control and eradication campaigns in order to assure reability of reagents and comparability of data on tuberculin testing (Kantor & Ritacco, 2006). Once positive BTB cattle is found, they need to be eliminated through of an official compensation for culled animals; nevertheless, if a political will is not established, this can represents a serious drawback that could jeopardize the control program. The first step is motivate farmers to cooperate, and convince them that progress is achievable, the farmer can be the leadership in programs to ensure that the farming community contributes fully to the design of control measures and their enforcement (Morris *et al.*, 1994).

In human population, pasteurization of milk and vaccination with bacillus Calmette-Guerin (BCG) prevents the infection, especially in children (Lee *et al.*, 2004). In Latin America, the milk is pasteurized but the quality control of this process is not always complete and reliable and its coverage is low in some areas (Ritacco *et al.*, 2006). Vaccination is focused on infants and tuberculin-negative individuals anticipating exposure (Acha & Szyfres, 2001).

### **1.7 Eradication**

Once a control BTB program is implemented with success, the eradication plan could begin. Infected herds will be quarantined, and animals that have been in contact with reactors need to be traced. Only test-and-slaughter techniques are guaranteed to eradicate tuberculosis from domesticated animals. Nevertheless, the occurrence of *M. bovis* in wildlife reservoir hosts complicates eradication efforts. Where there is interplay between infection in wildlife and domestic animals, eradication of the disease becomes impractical (Morris *et al.*, 1994).

Slaughter surveillance with tracing of infected animals may be a more efficient use of resources, when the eradication is nearly complete. Sanitation and disinfection may reduce the spread of the agent within the herd; rodent control may also be advisable on affected farms. Barriers can be used around hay storage areas to prevent wildlife access. In addition, biosecurity measures on farms decrease interactions between wildlife and domesticated animals (Collins, 2006).

The BCG vaccine has been used in calves, but this practice is inappropriate in countries attempting to eradicate TB, because it interferes with the interpretation of the tuberculin test and other immunological tests to detect infected animals (Suazo *et al.*, 2003; OIE, 2006).

Effective bovine tuberculosis vaccines are not currently available for cattle; the new vaccines are being developed and tested, particularly for wildlife reservoirs (Buddle *et al.*, 2006).

## **CHAPTER 2 – OBJECTIVES**

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**General objectives**

The main objectives of this thesis were to provide data on the epidemiologic situation of bovine tuberculosis (BTB) among dairy cattle in northern Ecuador and to compare the performances of the standard diagnostic tools used to identify *M. bovis*.

**Specific objectives:**

1. To evaluate the situation of BTB, in northern Ecuador (Mejia canton), among small, medium and large farms by using the single tuberculin caudal test and the comparative tuberculin test, and to isolate *M. bovis* from dairy cattle in this region.
2. To determine the prevalence and annual incidence of BTB in dairy cattle in this canton at individual and herd levels and to describe the associated risk factors.
3. To compare currently available laboratory tools to diagnose BTB and to characterize the distribution of lesions from slaughtered cattle as a marker for the possible route of transmission.
4. To perform a molecular characterization of *M. bovis* using spoligotyping and to compare the strains isolated from the Mejia canton.
5. To determine the zoonotic impact of BTB through a preliminary study in risk populations (farm and slaughterhouse workers) by tuberculin skin test.



## **CHAPTER 3 – RESULTS**

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### **3.1 Preliminary observations on *Mycobacterium* spp. in dairy cattle in Ecuador**

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## PRELIMINARY OBSERVATIONS ON *MYCOBACTERIUM* SPP. IN DAIRY CATTLE IN ECUADOR

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**Abstract.** This study evaluated bovine tuberculosis in Mejia canton, a major dairy cattle production region in Ecuador. Randomly selected cattle (1,012 from 59 farms) classified according to herd size were tested by the single tuberculin test (STT). Sixty days later, positive reactors were tested again by the comparative tuberculin test (CTT). In addition, tissue samples from two STT-CTT-positive reactors detected on a farm were obtained in a local slaughterhouse and analyzed bacteriologically. A total of 4.24% of the cattle were positive in the STT and 3.85% were positive in the CTT, with the highest number (7.95%) in large herds versus 3.4% in medium herds and 0.3% in small herds. *Mycobacterium bovis* was isolated from mesenteric lymph nodes and lungs of one animal. A 16S ribosomal RNA-based polymerase chain reaction confirmed culture results and differentiated mycobacteria other than *M. tuberculosis*. This study confirms the zoonotic importance of tuberculosis in Ecuadorian dairy cattle with herd size likely to be a crucial parameter in the prevalence of the disease. The implementation of a national control program is necessary and should be based on the detection of positive cattle by STT in combination with CTT.

### INTRODUCTION

Bovine tuberculosis (BTB) is an important zoonotic disease that has a worldwide distribution. The disease is caused by *Mycobacterium bovis* and apart from cattle, domestic animals and wildlife can occasionally be infected.<sup>1,2</sup> In developing countries in Africa, Asia, Latin America, and the Caribbean, where dairy production industry is a priority, intensification of the dairy industry has favored the transmission of the disease because proper standards for controlling BTB are often lacking. Thus, BTB is still causing severe economic losses in livestock due to loss of production, mortality, and condemnation of carcasses.<sup>3</sup>

Cattle become infected mainly by respiratory route and remain asymptomatic during the first few months after infection, but symptoms can appear when the delicate balance between the host and the infectious agent is lost because of stress factors such as immunosuppression or malnutrition.<sup>1</sup> After infection, nodular granulomas, known as tubercles, start to develop; these can occur in any tissue but are most frequently observed in lymph nodes, lungs, intestines, liver, spleen, pleura, and peritoneum. Clinical evidence of TB is usually lacking, and symptoms, if present, are not specific, e.g., sub-febrile temperatures, coughing, fatigue, loss of appetite, and reduced milk production.<sup>4</sup>

The status of BTB in Ecuador is not documented or clearly quantified because of several factors. These include lack of proper recording of positive cases, limited use of diagnostic tests, and insufficient veterinary inspection in most of the slaughterhouses. Isolated surveys carried out on BTB in cattle report a prevalence of 3.91% in the northern part of the country (Andino-Ashqui, unpublished data). In 2001, 46.55 cases of human TB per 100,000 inhabitants were reported by the Ministry of Public Health.<sup>5</sup> Nevertheless, studies have not been conducted to quantify cases of human TB caused by *M.*

*bovis*. In developing countries, the frequency and involvement of *M. bovis* in non-pulmonary TB is largely unknown because of limited laboratory facilities for culture and identification of tubercle bacilli.<sup>6</sup>

The standard method used for routine diagnosis of BTB is the tuberculin test,<sup>7</sup> which consists of reading the skin reaction to an intradermally injected purified protein derivative (PPD). Differentiation of the causative *Mycobacterium* species usually requires *in vitro* growth of the organism. Alternatively, the polymerase chain reaction (PCR) has been used with considerable success,<sup>8</sup> and has significantly reduced the time needed to confirm diagnosis in suspected cases. The purpose of this study was to evaluate the situation of BTB in Mejia canton, a major dairy cattle production region in Ecuador.

### MATERIALS AND METHODS

**Study design.** This study consisted of two parts: a field study in 2003 in Mejia canton, located in the Pichincha Province of Ecuador, followed by microbiologic analyses in Antwerp, Belgium. The field study was composed of a primary screening by a single bovine tuberculin test (STT) intradermally in randomly selected animals from three types of farms. Positively reacting animals were tested again with bovine and avian tuberculin by a comparative tuberculin test (CTT). Follow-up of skin-positive animals to the slaughterhouse was impossible for most animals. Therefore, samples from suspected bovine organs from the same region were collected in a slaughterhouse for further laboratory testing. Analyses of field samples were conducted at the Department of Microbiology, Unit Mycobacteriology, Institute of Tropical Medicine (Antwerp, Belgium). Laboratory studies included *in vitro* culture, microscopic analysis, and PCR.

**Tuberculin skin test.** A total of 1,012 randomly selected cross-bred dairy cattle improved by Holstein-Friesian cross-breeding from 59 farms were tested by an intradermal tuberculin skin test. Based on their herd size, dairy farms were grouped as large (more than 70 cattle), medium (25–70 cattle), and small (1–25 cattle). Tuberculin tests were restricted to animals more than six months of age, i.e., 22 ani-

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mals randomly selected from each large or medium farm and all animals from small farms. All animals were ear-tagged. Injection sites were cleaned and disinfected; swellings were measured with callipers immediately after inoculation and 72 hours later. A negative result in the SST was a swelling  $\leq 3$  mm, a suspicious result was a swelling between 3 and 5 mm, and a positive result was a swelling  $> 5$  mm.

To confirm positive or suspicious STT reactions, a comparative intradermal tuberculin test with bovine and avian tuberculin (CTT) was carried out after 60 days. The interpretation of the CTT after 72 hours is based on the comparison between the subsequent swellings. Reactions to bovine tuberculin  $\leq 5$  mm and  $> 4$  mm larger than the reaction to avian tuberculin were considered positive.

For the STT, 0.1 mL of mL bovine tuberculin (PPD-protein M. *bovis*, strain AN-5 at 25,000 IU/mL; CZ Veterinaria SA, Porriño Spain) was injected intradermally 7 cm from the base of the ventral side of the tail. The CTT injections were administered intradermally on the left side of the neck with the same bovine tuberculin and 0.1 mL of avian PPD tuberculin (0.5 mg/mL, 25,000 IU/mL; Servicio Nacional de Sanidad, Buenos Aires, Argentina) in previously shaven injection sites 12 cm apart.

**Isolation of *Mycobacterium* spp.** One hundred twenty-five biopsy specimens (lymph nodes, lungs, kidneys, and mammary glands) were taken from 40 cattle in the local district slaughterhouse with suspicious macroscopic lesions. In addition, 11 and 18 necropsy samples, respectively, were obtained from two cattle that were slaughtered (no. 65 and no. 1555) and positive in the CTT.

All specimens were stored in 2-mL Eppendorf (Hamburg, Germany) tubes containing Dubos broth (no. 0385-17-6; Difco Laboratories, Detroit, MI) supplemented with PANTA (Bactec PANTA Plus Kit no. 440 476 4; Becton Dickinson, Franklin Lakes, NJ) to preserve the mycobacteria and retard growth of contaminants.<sup>9</sup> Samples were transported at 5°C and stored at -20°C at the Department of Microbiology, Unit of Mycobacteriology, Institute for Tropical Medicine in Antwerp until processed.

***In vitro* culture and phenotypic identification.** All samples were decontaminated using the inverted Petroff method, i.e., 20-minute decontamination with 1 N HCl followed by alkaline neutralization (1 N NaOH).<sup>10</sup> Subsequently, Ziehl-Neelsen staining and inoculation into Löwenstein-Jensen and Stonebrink<sup>11</sup> media was conducted. *In vitro* identification of mycobacteria was carried out using biochemical (i.e., niacin production, nitrate reduction, urease production) and inhibition tests (thiophene-2-carboxylic hydrazide) and pigmentation and colony morphology as described by Lévy-Frébault and Portaels.<sup>12</sup>

**Polymerase chain reaction.** Necropsy samples from the two CTT-positive cattle were examined by PCR. A nested PCR was performed as described by Portaels and others<sup>13</sup> with primers for the 16S ribosomal RNA gene.<sup>14</sup> Primers P1: 5'-TGCTTAACACATGCAAGTCG-3' and P2: 5'-TGAGATTTCACGAACAACGC-3' were used at 56°C in the first PCR as general primers for identifying the *Mycobacterium* genus. Primers P3: 5'-AACCCGACCTTCGTGATG-3' and P9: 5'-CATGTCTTGTGGTGGAAAGCGC-3' were used at 66°C in the second PCR for specific identification of the *M. tuberculosis* complex. The final PCR product was approximately 540 basepairs.

DNA was extracted from tissues as reported by Portaels and others<sup>9</sup> using proteinase K. Briefly, 50  $\mu$ L of proteinase K (no. V3021, 20 mg/mL; Promega, Madison, WI) and 250  $\mu$ L of lysis buffer (1.6 M guanidine hydrochloride, 60 mM Tris, pH 7.4, 1% Triton, 60 mM EDTA, 10% Tween-20) were added to the decontaminated samples (250  $\mu$ L) and incubated for one hour at 60°C with shaking (200 rpm). Approximately 80 mg of glass beads was then added, samples were sonicated for five minutes at room temperature in a water bath sonicator (14 kHz; Branson 1200; Branson Ultrasonics Corporation, Danbury, CT), 40  $\mu$ L of acidified diatomaceous earth solution was added, and the suspensions were incubated for a maximum of two hours at 37°C with shaking (200 rpm). The suspensions were extracted twice with 70% ethanol and once with acetone with intermediate short centrifugations. The pellets were dried at 50°C in a heating block (Dri-Bath 16500; Thermolyne, Merck-Belgolo, Belgium) and resuspended in 90  $\mu$ L of TE buffer (10 mM Tris, 1 mM EDTA, pH 8), followed by incubation for 20 minutes at 55°C while shaking to obtain complete homogenization. The sample was briefly centrifuged and 50  $\mu$ L of supernatant was transferred to a new tube.

For the first PCR, 10  $\mu$ L of the DNA extract was added to 40  $\mu$ L of PCR mixture containing 50 pmol of each primer, 1 unit of Ampli *Taq* DNA polymerase (no. M1668; Promega), 200  $\mu$ M dNTP (no. 27-2094-02; Pharmacia, Uppsala, Sweden), 25  $\mu$ L of buffer, and 7  $\mu$ L of milli-Q water (Millipore, Billerica, MA) and overlaid with two drops of mineral oil (no. 50138; ICN, Costa Mesa, CA). A negative control (milli-Q water) and positive control (DNA at a specific concentration) were included in each PCR. Denaturation was at 94°C for 5 minutes, amplification for 40 cycles at 94°C for 45 seconds, 56°C for 45 seconds, and 72°C for 45 seconds, and final extension at 72°C for 10 minutes.

For the second PCR, 1  $\mu$ L of the first PCR product was amplified in a 25- $\mu$ L reaction mixture containing 25 pmol of each primer, 0.5 units of Ampli *Taq* DNA polymerase, 200  $\mu$ M dNTP, 12.5  $\mu$ L of buffer, 8.5  $\mu$ L of milli-Q water and overlaid with one drop of mineral oil. Amplification was composed of 25 cycles as described for the first PCR with an annealing temperature of 66°C. A total of 75  $\mu$ L of amplified DNA plus 2.5  $\mu$ L of loading buffer (Fermentas, St. Leon-Rot, Germany) and a molecular size marker were subjected to electrophoresis on a 2% agarose gel (no. EP-0010-10; Eurogentec, Seraing, Belgium) in 0.5% TAE buffer (1 mM EDTA, pH 8.0, 40 mM Tris-acetate). Bands were detected by staining with ethidium bromide (Bio-Rad Laboratories, Hercules, CA) and transillumination with ultraviolet light. The pattern of bands obtained was compared with those of negative and positive controls.

## RESULTS

The STT in 1,012 cattle showed 44 positive reactors (4.34%) distributed on large (26), medium (17), and small (1) farms, respectively, in addition to 14 suspected cases (1.38%) (Table 1). To confirm these results or discard possible false-positive results, the CTT was used 60 days after the STT and was conducted only with positive and suspected positive animals.

The CTT detected 39 positive cases among the STT-positive reactors. All five false-positives results were from



TABLE 1  
Results of the tuberculin test of 1,012 cattle in Mejía, Ecuador\*

Type of farm	No. of animals	No. of STT-positive animals (%)	No. of STT suspected animals (%)	No. of CTT-positive animals	No. of TB-positive farms/total no. of farms
Large	327	26 (7.95)	7 (2.14)	26 (7.95)	6/15
Medium	353	17 (4.82)	4 (1.13)	12 (3.40)	2/16
Small	332	1 (0.30)	3 (0.90)	1 (0.30)	1/28
Total	1,012	44 (4.34)	14 (1.38)	39 (3.85)	9/59

\* STT = single tuberculin test; CTT = comparative tuberculin test; TB = tuberculosis.

medium-sized farms (Table 1). These animals showed a strong reaction against both bovine and avian tuberculin, but the avian reaction was greater than the bovine reaction. All suspicious cases in the first test were negative in the second confirmative test. Thus, 3.85% of the animals were positive by tuberculin skin test.

Of 125 biopsy specimens obtained from the slaughterhouse, only five samples (from five different animals) yielded positive cultures. *In vitro* development was slow, i.e., it took 8–21 weeks before growth was visible and all cultures yielded exclusively mycobacteria other than *M. tuberculosis* (Table 2). None of the samples were positive by Ziehl-Neelsen staining.

*Mycobacterium bovis* was isolated from 4 of 29 necropsy samples from the two cattle that showed positive reactions in the CTT (Table 3). Ziehl-Neelsen staining of these samples showed mycobacteria in two mesenteric lymph nodes from both animals and in one lung.

The nested PCR for the 16S ribosomal RNA gene was conducted on the 29 specimens from two slaughtered cattle and confirmed the presence of *Mycobacterium* spp. in six samples. The PCR showed that only four organisms belonged to the *M. tuberculosis* complex (Table 3). Of three Ziehl-Neelsen-positive samples from the necropsy samples, two were identified by PCR as *M. bovis*, (both from the same animal), and the other as exclusively mycobacteria other than *M. tuberculosis* (Table 3).

## DISCUSSION

In most of Latin America, the zoonotic importance of BTB is not well quantified. Notable exceptions include Argentina and in Costa Rica, where international trade has resulted in close collaboration between governments and cattle owners associations, resulting in the control and elimination of BTB.<sup>6</sup> In Mexico, a high prevalence (> 4% by tuberculin skin test) is similar to that observed in Ecuador (3.85%), but is higher than the prevalence in Uruguay (0.5%) reported by Gil and Samartino.<sup>15</sup>

In previous isolated surveys in Ecuador, the STT and the CTT demonstrated variable prevalences of BTB over time and among various provinces ranging from 0.33% in Tungurahua in 1977 to 4.92% in the Pichincha Province in 2002 (Table 4). Results within the province of Pichincha also differed markedly. A prevalence of 2.80% was observed in Cayambe canton (Andino-Asqui O, unpublished data), which is located in the same province as the region in the present study. However, the results of our study were in sharp contrast to the prevalences of 0.43% (Torres L, unpublished data) and 0.47% (Salazar JC, unpublished data) in a survey of 18 herds in the same area. The 3.85% positive reactors found in the present study are consistent with the 4.92% obtained from 3,089 cattle sampled in Mejía canton on 13 large farms (Cano G, unpublished data).

The differences in prevalence found in relation to herd size are surprising (i.e., 7.95% and 3.40% on large and medium farms, respectively, and surprisingly only 0.3% on small farms). Although the limited number of animals investigated in this study does not allow firm conclusions to be made, higher risks for BTB in commercial (large) than in traditional (small) farms might be explained by closer contact between animals in the larger farms. Herd size as a decisive factor merits further investigation in view of the expansion of the dairy industry in Ecuador in recent years, which was caused by the high demand for milk and milk by-products. Furthermore, differences observed by other investigators should be related to the type of farms in the respective surveys.

Adams estimated that the sensitivity and specificity of the caudal fold single were 72% and 98.8%, respectively, whereas the sensitivity and specificity of the CTT with bovine and avian PPD was between 68.6% and 95% and 88.8% and 99.9%, respectively.<sup>16</sup> The rate of false-negative results is influenced by the time since exposure to environmental strains, immunosuppression, or anergic reactions in the early postpartum period. Desensitization can occur because of too short time intervals between tuberculin skin tests, errors in the pro-

TABLE 2  
Results of positive *in vitro* cultures from 125 biopsies collected in the slaughter house in Mejía, Ecuador\*

Sample no.	Type of sample	ZN	CM	P	N	NR	Ur	TCH	Final identification
BK-03-1445	Lung	–	R	N	–	–	–	+	MAIS
BK-03-1709	Lung	–	R	N	–	–	+	+	<i>M. gordonae</i>
BK-03-1712	Lung	–	S	N	–	+	–	+	<i>M. szulgai</i>
BK-03-1452	Lymph	–	S	N	–	–	–	+	<i>M. celatum</i>
BK-03-1719	Lung	–	R	N	–	–	–	+	MAIS

\* ZN = Ziehl-Neelsen; CM = colonial morphology; P = pigmentation; N = niacin production; NR = nitrate reduction; Ur = urease production; TCH = thiophene-2-carboxylic hydrazide; – = negative; R = rough; N = non-chromogenic; + = positive; MAIS = *Mycobacterium avium-intracellulare-scrofulaceum*; S = smooth.

## MYCOBACTERIUM SPP. IN DAIRY CATTLE IN ECUADOR

321

TABLE 3

PCR-positive samples using 16S ribosomal RNA gene from two tuberculin-positive reactors in comparison with Ziehl-Neelsen and *in vitro* culture\*

Animal no.	Sample no.	Type of biopsy	ZN	PCR		<i>In vitro</i> culture
				Msp	MTC	
65	BK-04-355	Lung	–	+	–	NG
65	BK-04-359	Mesenteric lymph node	–	+	+	<i>Mycobacterium bovis</i>
65	BK-04-360	Mesenteric lymph node	+	+	–	NG
1555	BK-04-410	Lung	+	+	+	<i>Mycobacterium bovis</i>
1555	BK-04-411	Mesenteric lymph node	+	+	+	<i>Mycobacterium bovis</i>
1555	BK-04-413	Mesenteric lymph node	–	+	+	<i>Mycobacterium bovis</i>

\* PCR = polymerase chain reaction; ZN = Ziehl-Neelsen; Msp = *Mycobacterium* sp., MTC = *Mycobacterium tuberculosis* complex; – = negative; + = positive; NG = no growth.

cedure, e.g., injection of insufficient tuberculin, use of tuberculin of reduced potency, and test and variability among observers.<sup>17</sup> Therefore, in the present study as a precaution, an animal was declared positive when its reaction versus bovine tuberculin was at least 4 mm bigger than the reaction to avian PPD, which is a more rigorous criterion in comparison with the standard used by the United States Department of Agriculture.<sup>18</sup>

False-positive reactions may be caused by sensitizations as a result of exposure to *M. avium*, *M. paratuberculosis*, environmental mycobacteria, and skin tuberculosis caused by slow-growing mycobacteria (*M. marinum*, *M. ulcerans*, *M. kansasii*, *M. avium-intracellulare*, and *M. scrofulaceum*) and rapidly growing mycobacteria (*M. fortuitum* and *M. chelonae*<sup>19</sup>). Other factors include the presence of *Corynebacterium*, *Fasciola hepatica*, and some *Nocardia* species, which pose problems in several countries.<sup>20</sup> In cattle vaccinated against paratuberculosis, the interpretation of single tuberculin tests might be difficult, but differentiation is possible by the use of the CTT.<sup>21</sup> *Mycobacterium avium* has frequently been recovered from cattle, causing only non-progressive lesions in the mesenteric lymph nodes.<sup>22</sup> Sensitization is often caused by exposure to infected domestic or wild birds, and occasionally by exposure to pigs infected with the *M. avium-intracellulare-scrofulaceum* complex, in which animals show a high reaction to avian PPD, as was observed in five animals from medium-sized farms. Therefore, a positive result in the CCT was restricted to those reactions where bovine tuberculin caused a minimum swelling of 5 mm and differed by at least 4 mm from

the swelling caused by the avian tuberculin; the same criterion was used in previous studies in Ecuador (Andino-Ashqui O, unpublished data and Cano G, unpublished data). Although STT and CTT were used in most of the previously performed surveys in Ecuador, the prevalence of aspecific reactions was not clearly mentioned and results referred to the animals positive only for bovine tuberculosis.<sup>3</sup>

*In vitro* cultures of 125 specimens from suspected cattle resulted in the isolation of mycobacteria in 5 samples (4%), all of which were mycobacteria other than *M. tuberculosis*. Thus, suspected postmortem lesions are not necessarily caused by *M. bovis* as reported by Quinn and others,<sup>23</sup> but may be caused by another pathogenic mycobacteria. In a survey in Argentina, bacilli in cattle from slaughterhouses were identified as *M. bovis*, *M. gastri*, *M. flavescens*, *M. phlei*, and *M. triviale*.<sup>24</sup> In Burundi, Rigouts and others isolated *M. bovis* from 15 of the 82 cattle sampled; 78% of those isolated grew on Stonebrink medium, which showed the preference of *M. bovis* for this medium.<sup>25</sup> Also identified were mycobacteria other than *M. tuberculosis* (in descending order of frequency: *M. terrae*, *M. nonchromogenicum*, *M. intracellulare*, *M. goodii*, *M. sp. rapid grower*, and *M. paratuberculosis*).

The isolation of *M. bovis* from only 4 of the 29 samples from two positive reactors with a high positive response in the tuberculin test demonstrates the difficulty related to the *in vitro* culture of this species because the distribution of the bacilli in lesions is not homogeneous.<sup>10</sup> Another factor that could have contributed to this is the stage of the disease at the time of sampling. Therefore, this result shows the need for a

TABLE 4  
Results of previous surveys on bovine tuberculosis in Ecuador (1977–2003)\*

Authors†	Year	Province	Test	No. of animals tested	% Positive for bovine tuberculosis
Acosta	1977	Tungurahua	STT-CTT	2,132	0.33
Torres	1996	Pichincha	STT	4,888	0.43
Salazar	2002	Pichincha	STT-CTT	3,006	0.47
Andino-Ashqui	2001	Pichincha/Imbabura/Carchi	STT-CTT	178	2.80
				329	7.29
				516	2.13
				1,023 (total)	3.91 (total)
Cano	2002	Pichincha	STT-CTT	3,089	4.92
Burbano	2002	Carchi	STT-CTT	3,011	1.73
Bedón	2003	Imbabura	STT-CTT	3,005	2.43
Alemán	2003	Tungurahua	STT-CTT	4,012	1.22

\* STT = single tuberculin test; CTT = comparative tuberculin test.

† Data are from doctoral theses.



detailed investigation to avoid false-negative cases. Furthermore, it has been shown that freezing-thawing cycles decrease the viability of mycobacteria.<sup>26</sup>

Although direct identification of acid-fast organisms by microscopy is fast, it does not identify the *M. species*.<sup>27</sup> In the present study, characteristics that identify *M. bovis*, such as acid-fast staining, rough morphology of the colony, susceptibility to thiophene-2-carboxylic acid hydrazide, and the absence of niacin production,<sup>25</sup> showed the usefulness of *in vitro* identification, especially when the PCR is not feasible. Microscopic examination by Ziehl-Neelsen staining in 125 specimens from the slaughterhouse and in 29 specimens from the two positives reactors showed the bacilli in only three samples. The low sensitivity of this method is well-known<sup>14</sup> because large numbers of organisms ( $> 10^4/\text{mL}$ ) must be present to make detection reliable.

All positive results obtained by conventional methods have been confirmed by PCR. The advantage of the PCR is that these results were obtained after 48 hours, whereas *in vitro* identification required 6–8 weeks. In addition, the PCR can determine whether the organisms belong to the *M. tuberculosis* complex, which requires another week by conventional methods. Since less optimal conditions during transport may have adversely affected the culture results, analysis immediately after sample collection is recommended.

The prevalences observed in the present study confirm the importance of bovine tuberculosis in Mejía canton and stress the need for surveillance at the national level. Use of diagnostic tools such as intradermal tests, *in vitro* cultures, and PCR will be useful in identifying reactors in herds, increasing epidemiologic information, and differentiating causative species. These tools will increase understanding of the zoonotic consequences, including the possible relation with herd size, and help to implement the most efficient control measures. Whether the PCR as a diagnostic tool is the most useful technique for rapid detection of *M. bovis* will require further evaluation on a larger sample size. In Ecuador, this technique is still too expensive for routine use. Thus, the STT and CTT in the field and use of *in vitro* cultures in the laboratory to identify the cause of BTB within a herd is recommended.

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### **3.2 Comparative intradermal tuberculin test in dairy cattle in the north of Ecuador and risk factors associated with bovine tuberculosis**

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## Comparative Intradermal Tuberculin Test in Dairy Cattle in the North of Ecuador and Risk Factors Associated with Bovine Tuberculosis

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**Abstract.** We studied the prevalence of bovine tuberculosis (BTB) in Mejia canton, the major dairy cattle production area in northern Ecuador. Twenty dairy herds comprising 2,022 cattle were selected. In 2007, each animal was tested using the comparative intradermal tuberculin test (CITT). In 2008, a follow-up test was performed in the same herds. The true annual incidence was 1.70%, and the true prevalence was 7.41% and 7.13% in 2007 and 2008, respectively. The prevalence was 0.27% and 0.57% in medium-sized herds in 2007 and 2008, respectively, compared with 8.63% and 8.43% in large herds ( $P < 0.01$ ). The number of skin test–positive cases also increased significantly with age ( $P = 0.03$ ), contacts with other species of animals ( $P < 0.01$ ), and introduction of new cattle ( $P = 0.04$ ). Herd prevalence was 55% in 2007 and 65% in 2008. This study shows the lack of knowledge in cattle farmers about this zoonosis and the necessity for a national BTB control program in Ecuador.

### INTRODUCTION

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis*, is a disease that mainly affects cattle. However, humans, domestic animals, and wildlife can also be infected and have severe symptoms.<sup>1</sup> BTB is considered by the World Organization for Animal Health (OIE) to be an important zoonotic disease of major socio-economic and public health importance, with an impact on international trade of animals and animal products.

In dairy cattle, the disease causes weight loss (36%), decreased milk production (13%), and lowered reproductive rate (12%).<sup>2</sup> The costs of diagnosis and treatment of cattle and humans and the costs of correct disposal of infected animal carcasses have an additional impact.

BTB is re-emerging in a number of developed countries because of environmental changes, the movement of people and animals, closer inter-species contacts, and changes in animal management.<sup>3</sup>

The prevalence and distribution of BTB in Latin America is poorly understood. The disease is officially endemic to 7 of the 34 countries in Latin America. Prevalence is high only in the Dominican Republic, 12 countries have reported as sporadic/low occurrence of BTB, and there are no data for the remainder.<sup>4</sup> It is estimated that 24% of the Latin American cattle population is unprotected against BTB<sup>5</sup> and 70% of the ~374 million cattle in Latin America and the Caribbean are held in areas where rates of *M. bovis* infection in cattle are  $> 1\%$ .<sup>6</sup> Ecuador is one of the countries with a relatively high prevalence, yet no official reports on the status of the disease are available.<sup>6</sup> A previous study in the same region estimated an overall prevalence of 3.85% in 1,012 cattle.<sup>7</sup> The Ecuadorian Service of Animal Health has no monitoring program, and economic losses caused by BTB have not been estimated.

The objectives of this epidemiologic study were to determine the prevalence and incidence of BTB among dairy cattle

herds in the Mejia canton, the main dairy production region in the north of Ecuador, and to describe the associated risk factors.

### MATERIALS AND METHODS

**Study area.** The study was carried out in five of the eight districts in the Mejia canton (1,459 km<sup>2</sup>), in Pichincha province in northern Ecuador. This area is located between 0°27' S and 78°25' W, with a mean altitude of 2,700 m (range, 600–4,750 m). There are in excess of 55,000 cattle in the canton,<sup>8</sup> with ~85% being dairy cattle.

**Animals.** Thirteen large herds ( $> 70$  animals) and seven medium herds (between 25 and 70 animals) were included in this study, covering a total population of 2,022 cattle. Herd size criteria were arbitrarily fixed. Individual herds were selected by geographic distribution and included after agreement by the farmer. Ninety percent of the cattle tested were Holstein Friesian, the rest were Jersey, Aberdeen Angus, Normando, Brown Swiss, and cross-bred animals.

To avoid confounding effects known to occur in young animals, skin tests were performed in all cattle  $> 6$  months of age. Cows 1 month before and 1 month after partus were also excluded because the interpretation of comparative intradermal tuberculin test (CITT) is also difficult in these cattle. Skin tests were applied in 2007, and a follow-up study was carried out in the same herds in 2008 to evaluate the true and estimated prevalence and incidence at individual and at herd level.

**CITT.** The CITT was used as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.<sup>9</sup> Briefly, it is based on the simultaneous use of bovine purified protein derivative AN5 strain (PPD-B) 20,000 IU/mL (Lot 06005) and avian-purified protein derivative D4 ER strain (PPD-A) 25,000 IU/mL (Lot 05001) from Symbiotics (Lyon, France).

The test was applied in the middle third of the neck of each animal; two different spots were shaved and cleaned at least 12 cm apart; the thickness of the skin was measured; and avian (PPD-A) and bovine (PPD-B) antigens were injected (i.e., 0.1 mL of PPD-B was injected in one shaved area and 0.1 mL of PPD-A was injected in the other shaved area). Skin

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thicknesses were measured again with calipers (Hauptner Herberholz, Germany) equipped with a spring to avoid manual pressure 72 hours after PPD injections. Measurements were always carried out by the same researcher.

The interpretation of the CITT is based on the observation that *M. bovis*-infected cattle develop a greater response to PPD-B than to PPD-A, whereas infection with *M. avium* or other mycobacteria induce the opposite reaction. A bovine reactor is therefore defined as an animal in which the relative increase in skin thickness at the injection site for PPD-B is at least 4 mm greater than the increase in skin thickness at the injection site for PPD-A. A negative reactor is identified when there is no reaction to the bovine antigen or when the difference of the skin thickness at the injection sites does not exceed 2 mm. An inconclusive reaction was recorded if reaction to both PPD-B and PPD-A exceeded 2 mm, but the difference between the bovine and avian reaction was < 4 mm. To evaluate whether an animal was classified as a positive or negative avian reactor, we applied the same parameters for PPD-A interpretation.

The result was plotted on a standardized comparative cervical tuberculin graph.<sup>10</sup> In accordance with international standards, inconclusive cases were tested again after at least 60 days to avoid the 'booster effect' of repeat PPD testing. The same scoring system was applied for this repeated testing. Animals that again had an inconclusive result were classified as "inconclusive" and therefore not considered as bovine reactors. All bovine reactors were marked using plastic ear-rings, and farmers/managers were advised to keep them separated from the rest of the herd to avoid the spread of the infection.

**Questionnaire survey.** To determine the possible risk factors related to the occurrence of BTB, each herd manager/owner was interviewed with the aid of a structured questionnaire. The questionnaire was divided into four parts comprising (1) general information about the farm, (2) livestock husbandry procedures, (3) symptoms in animals, and (4) previous diagnosis and history of the cases.

**Statistical analysis.** To account for the variability that is likely to exist between farms, CITT prevalence and confidence

intervals (CIs) were analyzed using estimators of cluster sampling. The true prevalence and incidence estimates in both years were calculated using the Rogan and Gladen equation (1978) under a Bayesian framework,<sup>11</sup> which considered the sensitivity (Se) and specificity (Sp) recorded for the skin test.<sup>12</sup> This analysis enabled the determination of the Se and Sp of the CITT.

CITT results in animals were compared with data obtained per animal (lactation, age) and per farm (herd size, new cattle introduction, and contact with other species of animals). Multivariate logistic regression analysis was used to assess the relationship between risk factors and PPD-B reaction. This analysis was undertaken in Survey package R version 2.7.2 (Statistics Department of the University of Auckland, Auckland, New Zealand) to take into account cluster sampling. Relationships were evaluated using odds ratios (ORs) whenever possible. Statistical significance with 95% CIs was set at the 0.10 probability level assessed by *P* values. In addition,  $\chi^2$  tests were used to evaluate the association between PPD-A reaction response and age. We excluded all male cattle (*N* = 8) from the multivariate analysis to avoid bias related to lactation.

## RESULTS

Figure 1 shows the type of farms sampled and the respective number of reactors per district in 2008. This figure also shows the location of the main city (Machachi) of the Mejia canton.

The CITT results are presented in Table 1. The overall prevalence of BTB assessed by CITT in the 20 selected herds was 7.10% (95% CI = 0.00–14.72) in 2007 and 6.91% (95% CI = 0.70–13.07) in 2008 in the same herds. The responses of the reactors to both PPD-A and PPD-B were 3.73% and 4.74% (*P* < 0.01), respectively. Reactions to PPD-A only were seen in 4.08% and 2.68% of the cattle in 2007 and 2008, respectively (Table 1). In 2008, 39 new positive cases were detected by CITT, resulting in a true annual incidence of 1.70% (95% CI = 0.04–3.13). The true estimated prevalence was 7.41% (95% CI = 5.53–9.44) and 7.13% (95% CI = 4.88–9.10) in 2007 and 2008, respectively. Application of Rogan and Gladen's

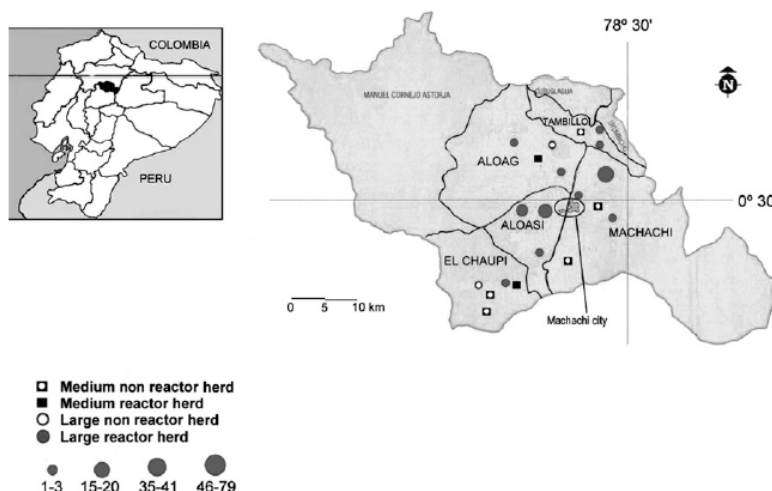


FIGURE 1. Study area with location of herds studied and estimation of bovine reactors per herd in the Mejia Canton of Ecuador in 2008.



TABLE 1  
Results of the comparative intradermal tuberculin test in relation to the herd size

Results of the comparative intradermal tuberculin test in relation to the herd size																	
Herd size*	No. of herds	Number of bovines in 2007							Number of bovines in 2008								
		Tested	Bovine reactors	Percent	Avian reactors	Percent	Inconclusive	Percent	Not read	Tested	Bovine reactors	Percent	Avian reactors	Percent	Inconclusive	Percent	Not read
Medium	7	369	1	0.27	15	4.08	14	3.79	1	354	2	0.57	13	3.75	17	4.89	7
Large	13	1,653	142	8.63	67	4.08	61	3.71	9	1479	122	8.43	35	2.42	68	4.70	33
Total	20	2,022	143	7.10	82	4.08	75	3.73	10	1833	124	6.91	48	2.68	85	4.74	40

Bovine reactor is PPD-B reaction  $\geq 4$  mm compared with PPD-A; avian reactor is PPD-A reaction  $\geq 4$  mm compared with PPD-B; and inconclusive is PPD-A and PPD-B reactions  $> 2$  mm and difference between PPD-B and PPD-A  $< 4$  mm.

\* Large herd =  $> 70$  bovines; medium herd = between 25 and 70 bovines.

equation showed 85% sensitivity and 99% specificity of the skin test used in this study (95% CI = 74–94 and 95% CI = 97–99.8, respectively).

There was a clear distinction between districts with a high prevalence and districts with a low prevalence. Few cases were recorded in the districts of Aloag, Chaupi, and Tambillo against high prevalences in the Aloasi (15.36% and 19.73%) and Machachi (16.19% and 12.23%) districts (Table 2).

In 2007, in 11 of the 20 (55%) herds tested, at least one bovine reactor was found (1 from medium and 10 from large herds), whereas in 2008, 13 of the 20 (65%) herds were positive (2 medium and 11 large herds; Figure 1).

Analysis of CITT results in relation to herd size showed an important difference between medium and large herds, with a BTB prevalence of 0.27% and 8.63%, respectively, in 2007 and 0.57% and 8.43% in 2008 (Table 1). Herd size proved to be a significant risk factor, with large herds having a much higher prevalence of infection ( $P < 0.01$ ).

Multivariate logistic regression analysis showed that lactating cows had a higher risk to be a bovine reactor in large than in medium herds (OR = 1.75). A significant positive correlation between bovine PPD reaction and the age of the animals was found ( $P = 0.03$ ; Figure 2; Table 3). On the contrary, the avian PPD response in cattle decreased with age ( $P = 0.02$ ; data not shown). Young cattle ( $< 2$  years) living in a large herd had a reduced risk compared with lactating cows from the same farm (OR = 19.48). Contact with other species of animals such as carnivores, small ruminants, pigs, or wildlife was also an important risk factor ( $P < 0.01$ , OR = 20.51). Introduction of new cattle was a less significant factor ( $P = 0.04$ , OR = 5.66). Cattle breed was not included in the data analysis because 90% were Holstein Friesian (Table 3).

## DISCUSSION

**Prevalence and incidence of BTB.** The tuberculin skin test is the internationally recognized standard method to identify infection with *M. bovis*, and it is currently the most widely applied screening test for detecting BTB in living animals. The

Se and Sp of the tuberculin skin test have been calculated to be between 77–95% and 98–99.9%, respectively.<sup>12</sup> However, the sensitivity can be affected by the potency and dose of tuberculin administered, the post-infection interval, desensitization, deliberate interference, post-partum immunosuppression, and variation in the interpretation by the observer. Contact with environmental non-tuberculous mycobacteria (NTM) or skin tuberculosis has also been documented to affect both the Se and Sp of the tuberculin test.<sup>13</sup> In this study, we took precautions to minimize factors influencing the skin test result by carefully applying the CITT and using high-quality, well-maintained PPD products. Readings before and after injections were done by the same person, and animals  $< 6$  or 1 month peri-partum were excluded.

The overall apparent prevalence of BTB differed slightly between the two surveys, i.e., 7.10% and 6.91% in the same group of animals in 2007 and 2008, respectively. However, the estimated true prevalence, obtained through the Bayesian model that also considered the Se and Sp of the CITT and included all infected animals, was found to be slightly higher: 7.41% and 7.13% in 2007 and 2008, respectively. This prevalence is higher than the overall prevalence of 3.85% reported in a previous study in the same region in 2003.<sup>7</sup> This difference could be attributed to the fact that the study of 2003 involved a higher number of small farms, which are known to have a lower prevalence of BTB.<sup>7</sup>

Our results might be an underestimate or overestimate of the true prevalence. We observed a strong association between the avian and the bovine PPD response ( $P < 0.01$ ), with 3.73% and 4.74% of the PPD-B reactors also reacting to PPD-A. Because these inconclusive cases were not considered BTB positive our results may be an underestimate of the true response. On the other hand, environmental NTM could increase the relative frequency and number of false-positive skin test reactors,<sup>14</sup> and strong avian reactions may affect the specificity of the test, which stresses the need for reviewing the interpretation of the tuberculin test in pastoral areas.<sup>15,16</sup>

In our surveys, 55% and 65% of herds were reactor herds in 2007 and 2008, respectively. It was considered a reactor herd

TABLE 2  
Apparent prevalence of bovine tuberculosis by CITT in five districts of the Mejia canton

District	Number of bovines in 2007					Number of bovines in 2008				
	Bovine reactors	Not read	Total	Prevalence (%)	95% CI	Bovine reactors	Not read	Total	Prevalence (%)	95% CI
Aloag	2	4	466	0.43	0.04–0.82	4	5	429	0.94	0.31–1.57
Aloasi	55	1	359	15.36	1.62–29.10	60	7	311	19.73	1.05–38.81
Chaupi	4	1	352	1.14	0.00–2.79	5	5	340	1.49	0.00–3.74
Machachi	80	1	495	16.19	0.00–39.95	52	16	441	12.23	0.00–28.69
Tambillo	2	3	350	0.57	0.31–0.84	3	7	312	0.98	0.00–2.00
Total	143	10	2,022	7.10	0.00–14.72	124	40	1,833	6.91	0.7–13.07

CITT = comparative intradermal tuberculin test.  
CI = confidence interval.

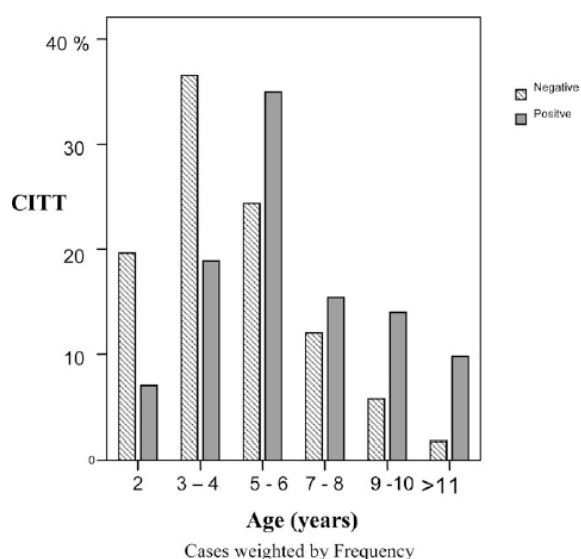


FIGURE 2. Distribution of bovine reactors to the CITT per age.

when one or more bovine reactors are found in a herd. About one half of those were large herds (10 of 20 in 2007 and 11 of 20 in 2008). In Uganda, a high herd prevalence was also noticed (46.6%).<sup>17</sup> As in our study, both the herd and individual prevalence was high in southern Tanzania (51% and 13.2%, respectively),<sup>18</sup> Eritrea (41.7% and 14.5%, respectively),<sup>19</sup> and Zambia (33% and 7.4%, respectively),<sup>20</sup> whereas in northern Tanzania, among pastoral cattle, a moderate herd but low animal prevalence was observed (11.8% and 0.9%,

TABLE 3

Multivariable logistic regression analysis of the risk factors associated with BTB

Variables	Logistic regression parameters			
	Estimate	SE	P value	Odds ratio (95% CI)
Lactation				
Young cows	—	—	—	1
Lactating cows	2.97	0.96	0.01*	NA
Non-lactating cows	2.17	0.61	< 0.01†	NA
Herd size				
≤ 70 cows	—	—	—	1
> 70 cows	-8.34	1.10	< 0.01‡	NA
Age	0.22	0.09	0.03*	NA
Animals introduced into the herd				
No	—	—	—	1
Yes	1.73	0.77	0.04*	5.66 (1.26–25.44)
Contact with other species				
No	—	—	—	1
Yes	3.02	0.60	< 0.01‡	20.51 (6.38–65.92)
Interaction terms				
Young animals × large herd	—	—	—	1
Lactating cows × medium herd	7.78	1.02	< 0.01‡	NA
Non-lactating cows × medium herd	-2.78	0.54	< 0.01‡	NA
Intercept	-10.08	0.89	< 0.01‡	NA

Statistical significance code:

\* 0.01–0.05.

† 0.001–0.01.

‡ 0–0.001.

NA = not applicable.

respectively).<sup>21</sup> As in Latin America, no BTB control program is in place in most African countries.

CITT prevalence per district showed a clear variation in the geographic distribution of *M. bovis* infection with low prevalence (from 0.4% to 1.5%) in three districts and high prevalence (from 12.2% to 19.7%) in two districts, indicating a significant geographic variation. For this reason, we used cluster sampling to calculate the confidence intervals. No variations in the husbandry systems, breed, or climate conditions between these districts were observed. However, the difference between districts could be attributed to the location of the farms in Machachi and Aloasi districts; the majority of these farms were close to the main city of Machachi, in an area where the farm density is very high. Risk of transmission of TB is higher in where farm density is high and the vicinity of neighbors with confirmed BTB has already been mentioned as an important risk factor in a previous study.<sup>22</sup> On the contrary, reduced farm risk for TB may be observed when large natural open lands surround the area,<sup>23</sup> avoiding contact with cattle from other farms.

The true annual incidence calculated in this study was 1.70%, which is unlikely to be influenced by removal of reactor animals, especially because infected animals were not culled. Studies to estimate the annual incidence of BTB in Latin America are scarce. In Argentina, it was calculated that on an average dairy farm without BTB control measures, one infected animal will eventually transmit the disease to 2.2 cattle per year.<sup>6</sup> Low incidences, between 0% and 0.22%, were reported in the Czech Republic, Hungary, Poland, and Slovakia between 1990 and 1999.<sup>24</sup>

Control of BTB is mainly based on the accurate detection of infected animals and removal of reactors before they become infectious for other animals in the herd. A series of tuberculin tests and subsequent culling can be applied until the herd is free of BTB. This test and cull program has allowed controlling the disease in many developed countries.<sup>4</sup> However, in this study, on economic grounds, culling of infected cattle was impossible because Ecuador has not yet implemented a sanitation policy with official compensation for culled animals. All farmers were informed on the risks of keeping infected cattle. Culling of a number of animals including some positive reactors was done in only one large herd. The prevalence in this herd between the first and the second survey was reduced from 40.3% to 31.9%. On the other hand, the recommendation to separate bovine reactors that were detected during the first survey (ear-marked) from the rest of the herd was systematically denied by the owners for the expected direct economical loss, which might have led to an overall increase of the prevalence within the herds.

We observed smaller skin reactions in older cows, although post-mortem examination showed clear macroscopic lesions. This finding requires additional research, but it limits the suggestion of Collins<sup>25</sup> to prioritize the removal of cattle with exaggerated skin test reactions as a sanitary measure in developing countries.

Vaccination of cattle is currently not desirable because it interferes with the tuberculin skin test in the field.<sup>2</sup> Besides, in Ecuador and other Latin American countries, in the absence of an eradication program, vaccination makes little sense.

**Risk factors.** This study is the first risk factor assessment of BTB in Ecuador. Similar studies have been carried out in several other countries, mainly in Africa, to understand the epidemiology of the disease at individual and at herd levels.<sup>17,18,26</sup>



Analysis on individual dairy cattle showed that PPD-B reactions increased significantly with age ( $P = 0.03$ ). This was partly because older cattle, over a longer period of life, had a higher frequency of exposure to *M. bovis* than younger cattle. These data are in agreement with studies performed in Ethiopia<sup>27</sup> and Canada,<sup>28</sup> in which an increased incidence of BTB was observed with increasing age. However, we observed that overall skin reactions in older animals were smaller than in younger animals. This may have been because animals recently infected with *M. bovis* (i.e., 6 weeks to 3 months) show a reasonably typical and large reaction compared with infections > 6 months. In the latter, skin tests could be less sensitive,<sup>29</sup> or in the advanced stage of the disease, these animals may even become anergic.<sup>1,29</sup> Risk of a positive PPD reaction can also vary with the general condition of the animals.<sup>20,27</sup>

On the other hand, PPD-A reactions decreased significantly with age ( $P = 0.02$ ), probably because the *Mycobacterium avium* complex (MAC) is more prevalent in the environment than *M. bovis*, because of its higher resistance to environmental conditions.<sup>14</sup> Young animals are in contact with these environmental mycobacteria early in life, whereas contact with *M. bovis* increases gradually with age, explaining higher PPD-A reactions at a younger age.<sup>14</sup> A similar phenomenon is observed among children in industrialized countries with a low prevalence of TB, where *M. avium* and *M. intracellulare* are the most important species isolated in case of lymphadenitis caused by mycobacteria.<sup>30</sup>

Herd size was identified as a significant risk factor in both the 2007 and 2008 surveys, confirming the results of a previous study in the same area carried out in small, medium, and large herds.<sup>7</sup> Likewise, in Tanzania, herds with > 50 cattle had a significantly greater BTB prevalence ( $P = 0.02$ ),<sup>21</sup> and in Canada, herds with > 80 cattle had an increased BTB risk compared with herds with < 16 cattle (OR = 9.3).<sup>27</sup> Similar results were obtained in Eritrea<sup>19</sup> and Zambia.<sup>20</sup> In a study performed in Uganda, however, no association between herd size and positive reactions was observed. In the latter study, the association was probably masked by the effect of high between-herd interactions.<sup>17</sup>

Cattle-to-cattle transmission can be facilitated by close contact, poor ventilation systems in barns and sheds, and the herd density.<sup>31</sup> Bacteria are shed through feces, milk, discharging lesions, saliva, and urine; likewise, transmission happens by different routes.<sup>32</sup> In developed countries, *M. bovis* infection in cattle is mostly confined to the respiratory system; a single bacillus transported within a droplet is probably sufficient to establish infection in the bovine lung.<sup>33</sup> Some of these transmission routes can be controlled by appropriate husbandry,<sup>25,34</sup> and in this way, control of the disease into the herd is feasible. All farm workers were advised to apply these procedures by means of information campaigns at the end of the study.

The most dangerous spots for nose to nose or mouth to mouth contact between animals are at the salt supplementation and feeding points,<sup>35</sup> especially in large herds with small-sized feeders. Animals under intensive farming management and confined to spaces or corrals allowing close physical contact before and during milking are more stressed.<sup>36</sup> Such crowding was frequently observed in the study area, particularly in the largest farms.

Studies carried out in Mexico showed more TB infections in dairy than in beef cattle,<sup>37,38</sup> and similarly in this survey, lactation was identified as another risk factor. Similar findings, although less significant ( $P < 0.05$ ), have been reported from

Tanzania, with 14.6% of lactating cattle reacting to the CITT compared with 12.0% in non-lactating cows.<sup>18</sup> In Ecuador, farmers keep old milking cows in the herd, which increases the infection risk by *M. bovis* during their life.

In developing countries, it has been reported that bovine TB infects a higher proportion of dairy breeds (*Bos taurus*) than indigenous zebu cattle (*Bos indicus*) and cross-bred beef cattle.<sup>1</sup> Additionally, multiple logistic models showed that the presence of exotic breeds is associated with a high risk of BTB (OR = 5.70)<sup>19</sup> and compared with zebu the risk of BTB was more than twice in Holstein cattle (OR = 2.32).<sup>27</sup> In our study area, there were no *Bos indicus* and relation between breed and BTB infection was not performed.

Recent introduction of new animals into a herd from the market<sup>17</sup> or from herd to herd<sup>17,28,39,40</sup> is also a known risk factor for BTB. Our data analysis showed a significant relationship between such cattle introduction and BTB infection ( $P = 0.04$ ). However, introduction of cattle from a different herd is not a common practice in this area, especially not in large farms.

Other risk factors mentioned in other field studies are the presence of wildlife, positive history of previous BTB within the herd, and the local herd prevalence.<sup>41</sup> These factors were also investigated in this study. The presence of wildlife in the area was declared by the farmers, although it was never observed by us; the follow-up was limited to 2 years and because of the lack of systematic data collection before to our intervention.

The risk factors described here are not only related to cattle but can also equally contribute to the infection of *M. bovis* in humans, especially in at-risk populations in close contact with cattle and their products. In an independent study conducted in the same area (Benitez-Capristros and others, unpublished data), there was a significant association between positive skin reactions among farm workers and the presence of the disease in the herd, confirming the risk of exposure to *M. bovis* in those farms. Moreover, the probability for positive PPD response was almost three times higher in males ( $P = 0.04$ ; OR = 2.75). In developing countries, *M. bovis* is estimated to cause 2% of all human cases of TB,<sup>6</sup> and because the prevalence in cattle seems to rise because of the lack of a national control strategy, it might have a more significant impact on human TB in the long term. BTB control strategies should be based on prevention, control, and eradication; fundamental activities concerning animal husbandry, removal of known sources of infection, early diagnosis, quarantine, movement control, and environmental hygiene must be sound.<sup>22</sup> In further studies in this area, *in vitro* assays could be used in cattle such as the interferon- $\gamma$  assay. This method has a higher sensitivity and specificity compared with the skin test.<sup>42</sup> Unfortunately, because of its high cost, it can currently only be applied for research purposes in Ecuador.

Results from this study highlight the need for a national BTB control program in Ecuador, which should be supported by sanitary policies to allow the identification of the endemic areas nationwide, establishing animal movement restrictions, culling, sanitary slaughtering, compensation strategy for culling and laboratory tests to identify the *Mycobacterium* species and strains involved. In addition, education of farm workers about the disease could help prevent new cases in animals and humans and help improve the use of preventive measures.

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### **3.3 Post-mortem examination and laboratory based analysis for the diagnosis of bovine tuberculosis among dairy cattle in Ecuador**

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## Post-mortem examination and laboratory-based analysis for the diagnosis of bovine tuberculosis among dairy cattle in Ecuador

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### ABSTRACT

Veterinary inspection in slaughterhouses allows for the detection of macroscopic lesions reminiscent of bovine tuberculosis, but the presence of *Mycobacterium bovis* must be confirmed by laboratory methods. This study aimed at comparing the performances of the standard diagnostic tools used to identify *M. bovis* in tissue specimens sampled from suspicious animals. During a two years period, 1390 cattle were inspected at the Machachi abattoir in the Mejia canton – Ecuador. A total of 33 animals with granulomatous lesions were detected, representing 2.33% (16/687) and 2.42% (17/703) animals examined in 2007 and 2008, respectively. Ninety-four tissue specimens were sampled and screened for the presence of mycobacteria. Acid-fast bacilli were identified in one third of the suspicious cattle (11/33) and suggestive microscopic lesions in 27.3% (9/33) of the samples examined by direct microscopy and histopathology, respectively. Culturing on Stonebrink medium and 16S-rRNA-based polymerase chain reaction (PCR) yielded 36.4% (12/33) and 27.3% (9/33) of positives, respectively. Compared to culture, other diagnostic procedures displayed a lower sensitivity, with 56.5% for PCR, and 43.5% for direct microscopy and histopathology; however, the specificity was higher (94.4% for PCR and microscopy, and 97.2% for histopathology). We conclude that reliable post-mortem laboratory testing either requires the combination of a set of available diagnostic tools or necessitates the development of improved new-generation tools with better sensitivity and specificity characteristics.

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## 1. Introduction

Bovine tuberculosis (BTB) is present in most developing countries where surveillance and control activities are not or inadequately implemented (Cosivi et al., 1998). In Ecuador, there is no BTB control program in place. An apparent prevalence of 7.95% was observed among dairy cattle from large herds in 2004 (Proaño-Pérez et al., 2006), and it increased to 8.63% three years later in the same region (Proaño-Pérez et al., 2009). Herd size was identified as an important risk factor in this husbandry system, and the number of skin-test-positive cases also increased significantly with cattle age (Proaño-Pérez et al., 2009).

BTB control programs consist of pasteurization of milk, meat hygiene control through macroscopic inspection at slaughter (veterinary inspection), and skin testing to detect and cull infected animals (Collins, 2006).

Cattle are mainly infected with *M. bovis* through inhalation of aerosolized droplets (Goodchild and Clifton-Hadley, 2001). However, the process by which exposure to infection can lead to a range of disease outcomes is not fully understood (Neill et al., 1994; Pollock et al., 2006). Not all infected cattle display gross lesions, but if present, they are often located in thoracic lymph nodes (Whipple et al., 1996) even in apparently healthy cattle. Lesions due to non-tuberculous mycobacteria (NTM) can be easily mistaken for BTB lesions (Oloya et al., 2007). Consequently, laboratory approaches are important to confirm and identify the mycobacterial species involved.

Different methods can be used to identify the disease in humans and animals. Microscopic detection of mycobacteria is insensitive and does not permit the identification of the mycobacterial species involved. Culture of *M. bovis* can be more sensitive and allows for species identification, but is time consuming due to the slow growth of the bacilli (Mangiapan et al., 1996). Polymerase chain reaction (PCR) can reduce the time for detection and also identify the species.

The aim of this study was to compare currently available laboratory tools to diagnose BTB using abattoir samples taken during veterinary inspection, characterize the distribution of lesions observed in the organs of inspected cattle, and study the true prevalence of BTB.

## 2. Materials and methods

### 2.1. Study design

Veterinary inspection was carried out in 1390 slaughtered cattle from 112 different dairy farms at the abattoir of Machachi located in the Mejía canton (0°27'SL and 78°25'WL), located in the major dairy cattle production

area in northern Ecuador. There was no previous studies on BTB in this abattoir. In total, 29 interventions were performed at the slaughterhouse during the period from February to March in the years 2007 and 2008. Detailed post-mortem examination was performed in 687 and 703 cattle, respectively. In this study, all cattle were inspected during each intervention, and cattle originating from areas outside the Mejía canton were excluded. Individual data were recorded for each animal by the use of a questionnaire with a focus on age, breed, sex, name of the owner, origin of the animal and lesions found during the inspection. Five of the animals examined during the abattoir survey were previously identified as bovine comparative intradermal tuberculin test (CITT) reactors in a survey among dairy cattle in the Mejía canton (Proaño-Pérez et al., 2009).

### 2.2. Post-mortem examination

Detailed veterinary inspection was carried out on all cattle. The lungs, liver, spleen, kidney and mammary gland were palpated carefully and inspected externally and internally. Mandibular, retropharyngeal, tracheobronchial, mediastinal, hepatic, mesenteric and supramammary lymph nodes were sliced into thin sections (2–3 mm) and inspected *in situ* for detection of visible lesions.

### 2.3. Samples

Tissue specimens were taken from multiple organs using disinfected knives (i.e., chlorine 10%) from all suspected animals for further laboratory-based diagnosis. We included all organs with visible lesions, and added lung tissue taken from the inferior lobe in all sampled cattle, even if no macroscopic lesions were visible. In total, 94 tissue samples were collected from 33 suspected cattle (2–8 specimens/animal, depending on the lesions found); 65 of them were sampled from tissues with visible gross lesions suggestive for mycobacterial infection, and the rest were samples without lesions taken from lungs.

All samples were collected in individual sterile tubes, stored in a cool box, and transported the same day to the Laboratory of Microbiology at the International Centre of Zoonoses, in Quito. Upon arrival, processing of samples was carried out using aseptic techniques in a biosafety cabinet to avoid cross-contamination between samples. In order to perform microscopy, PCR, and *in vitro* culture, approximately 5 g of tissue were stored at –20 °C in 2 mL Eppendorf® tubes containing Dubos broth (No. 0385-17-6, Difco Laboratories, Detroit, USA) supplemented with PANTA (Bactec PANTA plus kit No. 440 476 4; Becton Dickinson, NJ, USA) to inhibit the growth of contaminants and to preserve the mycobacteria. During the international transport to Belgium the samples were packed in a cooler. All samples underwent 2 freeze–thaw cycles due to the storage and shipping prior to their final processing at the Mycobacteriology Unit, in the Institute of Tropical Medicine of Antwerp, Belgium. Duplicate samples that were stored in Ecuador had only one freeze–thaw cycle and were processed locally once the culture technique was implemented.

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In addition, about 1 cm<sup>3</sup> of each sample was stored in 10% formalin for histopathologic analyses at the Department of Morphology and Pathology from the Faculty of Veterinary Medicine in the University of Liege, Belgium.

#### 2.4. Microscopy and in vitro culture

Upon arrival in Antwerp, all samples for bacteriological examination were cut and homogenized in a sterile mortar with 2 mL of sterile phosphate buffered saline. *In vitro* culture was carried out after decontamination using the “reversed” Petroff method (Palomino and Portaels, 1998): 20 min incubation with 3 mL of 1 N HCl followed by neutralisation with 3 mL of 1 N NaOH and centrifugation for 20 min at 3000 × g. The pellet was mixed with 2 mL sterile distilled water and inoculated onto Löwenstein–Jensen (LJ) (Parks, 1997), and Stonebrink medium (i.e. LJ without glycerol but supplemented with 0.4% sodium pyruvate). Isolates were identified as *M. bovis* by spoligotyping characterized by a typical lack of spacers 3, 9, 16 and 37–42 (Kamerbeek et al., 1997). For Ziehl–Neelsen (ZN) staining and subsequent microscopic inspection to detect acid-fast bacilli (AFB), a slide was prepared using 1 drop of the processed specimen. Stained slides were examined with a normal light microscope using objective 100× and immersion oil.

#### 2.5. Polymerase chain reaction

DNA was extracted from the re-suspended pellet using the method developed by Mangiapan and colleagues (1996). A routinely-applied, nested PCR targeting the 16S ribosomal RNA gene was performed to detect DNA specific for the *M. tuberculosis*-complex using the following primers (Portaels et al., 1996): P1 (5′-TGCTTAACACATGCAAGTCG-3′) and P2 (5′-TCTCTAGACGCGTCCTGTGC-3′) for the first run, and P3 (5′-AACCCGGACCTTCGTCCATG-3′) with P9 (5′-CATGTCTTGTGGTGAAAGCGC-3′) for the second run. Samples were considered positive to bovine tuberculosis when a band of 500 bp was observed on a 2% (w/v) agarose gel.

#### 2.6. Histopathology

Slides for histopathology examination were prepared using standard techniques, and processed for standard hematoxylin–eosin (HE) and ZN staining. A sample was considered positive if lesions characteristic for BTB were demonstrated (granulomatous inflammation associated with focal caseous necrosis or mineralization), or if AFB were observed in the ZN stain (Whipple et al., 1996).

#### 2.7. Statistical analysis

The true prevalence (*TP*) based on detection of gross visible lesions was calculated using the Rogan–Gladen's Equation (1978) under a Bayesian modelling approaches (Berkvens et al., 2006). The Rogan–Gladen equation describes the estimation of *TP* through the apparent prevalence (*P*), and the *Se*, and *Sp* of a test [ $P = TPSe + (1 - TP)(1 - Sp)$ ]. Since there is no gold standard test, *TP* must be estimated imposing constraints on the

parameters (Berkvens et al., 2006). Bayesian approach lets to incorporate external information by specifying prior distributions on the parameters, i.e., prior knowledge and/or beliefs regarding to *Se* and *Sp* of necropsy (Enoe et al., 2000; Branscum et al., 2005). This approach makes possible inferences about *Se* and *Sp* with 95% probability interval (95%PI) of necropsy used in this study. The prior distribution on *Se* was elicited from a previous study that considers the most likely value (modal), thus the *Se* of necropsy was 0.8 with a 10th percentile of 0.55; which is acceptable for this test and was chosen because a similar procedure during the necropsy was performed in the same lymph nodes (Norby et al., 2004), and based on the same measures through veterinary inspection to discard carcasses in Ecuador (Personal communication). The prior distribution for the *Sp* was elicited from the experience of veterinarian inspectors from the slaughterhouse of the Mejia canton, taking into account the presence of other infectious diseases causing lesions in lymph nodes specially in dairy cattle, thus a modal value of 0.95 with a 5th percentile of 0.80 were set to model the uncertainty about *Sp*. The BetaBuster software (available at <http://www.epi.ucdavis.edu/diagnostictest/>) (Branscum et al., 2004) computed the two parameters of a Beta prior distributions based on the mode and one percentile for *Se* and *Sp*, i.e., Beta for *Se* (5.28, 2.07), and Beta for *Sp* (21.20, 2.06). A binomial model was built and Bayesian estimation was done through Gibbs sampling in WinBUGS (Spiegelhalter et al., 1996). A burn-in phase of 1000 iterations was used and the model was run for another 10,000 iterations to obtain estimates. The outcomes were mean and percentiles (i.e., 2.5 and 97.5) sampled from the posterior distributions of *TP*, *Se* and *Sp*. Three chains starting in different values were set and their convergence was analyzed through trace plot. Additionally the effect of different prior distributions for the parameters of the model was analyzed in order to evaluate how prior beliefs could affect the posterior estimates of *TP*, *Se* and *Sp*. Seven scenarios were built assuming prior different beliefs about the parameters (Table 1): (Scenario 1) No prior constraints: *TP* ~ Uniform (0, 1), *Se* ~ Uniform (0, 1) and *Sp* ~ Uniform (0, 1); (Scenario 2) Prior distributions for *Se* and *Sp* uniformly distributed according to ranges suggested by Norby et al. (2004), and expert opinion of veterinaries in slaughterhouses *TP* ~ Uniform (0, 1), *Se* ~ Uniform (0.51, 0.98) and *Sp* ~ Uniform (0.80, 0.90); (Scenario 3) True prevalence constrained uniformly to values less than 10% and *Se* and *Sp* with similar values to previous experiences *TP* ~ Uniform (0, 0.10), *Se* ~ Uniform (0.50, 1.00) and *Sp* ~ Uniform (0.70, 1.00); (Scenario 4) True prevalence constrained uniformly to values less than 5% and *Se* and *Sp* with similar values to previous experiences but wider: *TP* ~ Uniform (0, 0.05), *Se* ~ Uniform (0.40, 1.00) and *Sp* ~ Uniform (0.60, 1.00); (Scenario 5) Assuming that *Se* of the necropsy is 70% and *Sp* 80% but with high uncertainty *TP* ~ Uniform (0, 1.00), *Se* ~ Beta (8.00, 4.00) and *Sp* ~ Uniform (9.00, 2.00); Scenario 6) Assuming that *Se* of the necropsy is 70% and *Sp* 80% but with low uncertainty *TP* ~ Uniform (0, 1), *Se* ~ Beta (71.0, 31.0) and *Sp* ~ Uniform (86.0, 16.0); and finally (Scenario 7) *TP* ~ Uniform (0, 1), Beta (5.28, 2.07) and Beta (21.20, 2.06) which correspond to our beliefs about necropsies.

## Study 3

68

F. Proaño-Pérez et al. / Preventive Veterinary Medicine 101 (2011) 65–72

**Table 1**Estimates of parameters in the Bayesian binomial model using different prior distributions for *TP*, *Se* and *Sp* of necropsy to identify bovine tuberculosis.

Scenario	Prior distribution	Posterior mean %	IP 95%
Scenario 1	<i>TP</i> ~ Uniform (0, 1)	Did not converge	–
	<i>Se</i> ~ Uniform (0, 1)	Did not converge	–
	<i>Sp</i> ~ Uniform (0, 1)	Did not converge	–
Scenario 2	<i>TP</i> ~ Uniform (0, 1)	1.11	0.005–4.69
	<i>Se</i> ~ Uniform (0.51, 0.98)	71.89	68.80–94.60
	<i>Sp</i> ~ Uniform (0.80, 0.90)	98.22	98.08–99.64
Scenario 3	<i>TP</i> ~ Uniform (0, 0.10)	1.83	0.008–4.40
	<i>Se</i> ~ Uniform (0.5, 1.0)	72.04	50.87–98.26
	<i>Sp</i> ~ Uniform (0.7, 1.0)	98.74	97.27–99.99
Scenario 4	<i>TP</i> ~ Uniform (0, 0.05)	1.95	0.01–4.50
	<i>Se</i> ~ Uniform (0.4, 1.0)	66.11	41.0–97.7
	<i>Sp</i> ~ Uniform (0.6, 1.0)	98.71	97.7–99.8
Scenario 5	<i>TP</i> ~ Uniform (0, 1)	1.50	0.005–4.30
	<i>Se</i> ~ Beta (8.0, 4.0)	63.63	34.63–87.83
	<i>Sp</i> ~ Beta (9.0, 2.0)	98.31	97.05–99.65
Scenario 6	<i>TP</i> ~ Uniform (0, 1)	0.3	0.01–1.25
	<i>Se</i> ~ Uniform (71, 31)	69.28	60.01–78.03
	<i>Sp</i> ~ Uniform (86, 16)	96.86	95.00–97.7
Scenario 7	<i>TP</i> ~ Uniform (0, 1)	1.50	0.005–4.69
	<i>Se</i> ~ Uniform (5.28, 2.07)	67.02	68.8–94.6
	<i>Sp</i> ~ Uniform (21.20, 2.06)	98.34	97.08–99.64

The agreement among the different laboratory-based diagnostic methods applied in this study was assessed by Cohen *kappa* statistic under cluster sampling (*svykappa* function) under R environment, described by Lumley (2004), which is the approach used to assess different tests without assuming that one is the best. The results obtained were classified according to the Altman scale to give a grade of significance (i.e., >0.80: very good agreement, 0.61–0.80: good agreement, 0.41–0.60: moderate agreement, 0.21–0.40: fair agreement, and ≤0.20: poor agreement) (Abraira, 2000). In addition, the *Se* and *Sp* of histopathology, microscopy, and PCR were calculated taking the *in vitro* culture as a gold standard test. The 95% confident intervals (CI) were estimated for *Se* and *Sp* of each test.

### 3. Results

The apparent prevalence of gross visible lesions found by veterinary inspection was 2.33% (95% CI = 1.37–3.73) and 2.42% (95% CI = 1.41–3.84) for the 2007 and 2008 surveys respectively. The *TP* was calculated to be 1.50% (95% PI = 0.005–4.69) taking into account our prior beliefs related with the characteristic of this test. The estimated *Se* and *Sp* of necropsy in this study were 67.02% (95% PI = 29.39–94.58) and 98.34% (95% PI = 97.08–99.64), respectively. The estimates of the *TP* did not show important differences under several constraints, they were around 1.5%, although when constraints had low uncertainty (scenario 6) the estimates of *TP* was lower. The estimates for *Sp* of necropsy were in general consistent and high. The estimates for *Se* of necropsy varied according to the constraints and wide intervals were found (Table 1).

Macroscopically, we observed yellowish granulomatous tubercles in mediastinal (51.32%), tracheobronchial (23.68%), hepatic (11.84%), and retropharyngeal (9.21%) lymph nodes. Only one animal showed visible lung lesions. Also, the five CITT reactors showed visible lesions in thoracic lymph nodes, but not in the lungs.

From the 94 specimens, 31 yielded one or more positive results in the laboratory (Table 2): all 24 positive lymph nodes from 15 cattle showed visible lesions (8 bronchial, 7 mediastinal, 3 hepatic, 3 mammary, 2 retropharyngeal, and 1 scapular lymph node), versus only one of the 7 (14.28%) positive lung tissues (Table 2). Fourteen of 31 specimens yielded a positive result in only a single test, 6 scored positive in 2 tests, 6 in 3 tests, and 5 had a positive result in all 4 tests applied.

On the animal level, this resulted in the detection of *M. bovis* in 51.5% (17/33) of the suspected animals. In 7 animals, only one specimen was found positive, whereas the remaining animals had 2–4 positive specimens.

Histopathology examination allowed for the identification of BTB in 27.3% (9/33) of the suspected animals. It showed focal necrosis, dystrophic calcification and/or Langhans- and foreign body-type giant cells in a total of 12 biopsies. Giant cells were identified in the lung sample showing gross lesions and in 3 mediastinal lymph nodes. Necrosis and/or calcification were more frequently observed (11 samples). In the remaining animals (24/33), a variety of lesions was identified in lungs (chronic interstitial pneumonia 65.7%, atelectasis 17.1%, emphysema 8.6%, granulomatous pneumonia 5.7% and bronchioloalveolar carcinoma 2.9%), and lymph nodes (caseous lymphadenitis 38.3%, follicular hyperplasia 31.9%, lymphoma 8.5%, oedema with infiltrate of macrophages 6.3%, other lesions 4.2%, and no significant lesions 10.6%).

AFB were detected in ZN-stained smears from 33.3% (11/33) of suspected animals. Fourteen (14) of 94 smears were positive: 4 from lung samples and 10 from lymph nodes, mainly bronchial and mediastinal (Table 2). However, the number of AFB detected per smear was very low, with a maximum of 10. Five cases had more than 3 AFB per slide but in 9 slides only 1 or 2 AFB were observed.

*In vitro* culture proved the most sensitive method in this study. *M. bovis* was isolated from 36.4% (12/33) suspected cattle. Bacilli were grown in 23 samples after about 7 weeks, and on Stonebrink medium only. The colonies were

## Study 3

**Table 2**

Distribution of positive laboratory results for bovine tuberculosis observed in 31 specimens from 17 different animals taken from dairy cattle at the slaughterhouse of Machachi, Ecuador.

Area	Animal identification	Farm	Specimen	Visible lesion	Smear ZN	Culture ST	PCR 16S rRNA	Histo-pathology
Machachi	1	A	Bronchial LN	Yes	Positive	–	–	–
	5	B	Lung	No	–	–	Positive	–
	8	C	Bronchial LN	Yes	–	Positive	–	Positive
			Retropharyngeal LN	Yes	–	–	–	Positive
	33	C	Lung	No	–	Positive	–	–
	14	C	Retropharyngeal LN	Yes	–	Positive	–	Positive
			Bronchial LN	Yes	–	Positive	Positive	Positive
			Hepatic LN	Yes	–	Positive	Positive	–
			Mammary LN	Yes	–	Positive	Positive	–
			Lung	No	Positive	Positive	Positive	–
	23	C	Lung	No	Positive	–	–	–
			Mediastinal LN	Yes	–	Positive	–	–
	13	D	Hepatic LN	Yes	–	Positive	Positive	Positive
			Bronchial LN	Yes	–	Positive	–	–
			Lung	No	–	–	Positive	–
Tambillo	26	D	Mediastinal LN	Yes	Positive	Positive	Positive	Positive
	30	D	Mediastinal LN	Yes	–	Positive	–	–
	32	D	Mediastinal LN	Yes	–	–	–	Positive
	12	E	Bronchial LN	Yes	Positive	Positive	–	–
			Lung	No	Positive	Positive	Positive	Positive
Aloasi	25	F	Mammary LN	Yes	Positive	–	–	–
	16	G	Scapular LN	Yes	–	Positive	–	–
			Bronchial LN	Yes	Positive	Positive	Positive	–
Aloag			Mediastinal LN	Yes	–	Positive	–	Positive
	31	G	Mammary LN	Yes	Positive	–	–	–
	28	H	Bronchial LN	Yes	Positive	Positive	Positive	–
	19	I	Lung	Yes	Positive	Positive	Positive	Positive
			Mediastinal LN	Yes	Positive	Positive	Positive	Positive
Chaupi			Bronchial LN	Yes	Positive	Positive	Positive	–
			Hepatic LN	Yes	–	Positive	–	–
	20	J	Mediastinal LN	Yes	Positive	Positive	Positive	Positive

ZN = Ziehl-Neelsen, ST = stonebrink, LN = lymph node.

obtained from 19 lymph node and 4 lung specimens, all positive *M. bovis* isolates were confirmed by spoligotyping. No mycobacteria other than *M. bovis* were detected by culture.

Nested PCR detected 27.23% (9/33) positive animals. *M. tuberculosis* complex DNA was amplified in 15 of 94 samples: 10 lymph nodes and 5 lung biopsies (Table 2). Negative DNA-extraction and PCR controls yielded a negative result in all PCR runs.

Survey kappa analysis of the various laboratory tests revealed a substantial concordance between culture and PCR ( $\kappa = 0.61$ ), a moderate concordance between culture and histopathology ( $\kappa = 0.49$ ), culture and microscopy ( $\kappa = 0.44$ ), PCR and microscopy ( $\kappa = 0.47$ ), and PCR with histopathology ( $\kappa = 0.44$ ) (Table 3). A low concordance was observed between microscopy and histopathology ( $\kappa = 0.20$ ).

Compared to *in vitro* culture, the 3 remaining techniques had a low sensitivity to detect BTB. The Se of PCR (56.5%; 95% CI = 34.4–76.8) was higher than that of microscopy (43.5%; 95% CI = 23.2–65.5) and histopathology (43.5%; 95% CI = 23.2–65.5). Specificities were much higher with 94.4% (95% CI = 86.2–98.4) for PCR and microscopy, and 97.2% (95% CI = 90.2–99.6) for histopathology.

#### 4. Discussion

The prevalence of BTB, as determined by the detection of gross visible lesions in slaughtered animals, was similar in 2007 and 2008 (2.32% and 2.41%, respectively). However, the true prevalence, taking into account the Se (83.33%) (Norby et al., 2004) and Sp (95%) calculated for this method, was lower (1.50%). As this study was the first of its kind in the Mejia canton, we cannot compare our

**Table 3**

Concordance analysis by survey sampling Kappa of the laboratory tests applied to diagnose BTB from specimens taken at the abattoir of the Mejia canton—Ecuador during 2007 and 2008.

Tests	Microscopy		Culture		PCR		Histopathology	
	$\kappa$ value	Std error	$\kappa$ value	Std error	$\kappa$ value	Std error	$\kappa$ value	Std error
Microscopy	1	na						
Culture	0.44	0.12	1	na				
PCR	0.47	0.15	0.61	0.08	1	na		
Histopathology	0.20	0.14	0.48	0.06	0.44	0.13	1	na

$\kappa$ : Kappa, na: not applicable.



results with previous data of this region, even though it is the most important dairy region in the north of Ecuador. Abattoir surveys performed in other provinces showed a lower prevalence: 0.12% and 0.46% in Guayas, and 0.21% in Santo Domingo de los Sáchilas (Mata, 1973; Cueto and Suárez, 1993; Coloma, 2000). However, these studies – which were never officially published – were carried out in meat production regions and therefore are not comparable with our data from dairy cattle as shown by data from other countries. In Mexico, an abattoir survey revealed the presence of gross lesions typical for BTB in 16% of dairy cattle (Milian-Suazo et al., 2000), whereas another survey revealed significantly less gross lesions among beef cattle (0.05%) (Brown and de Anda, 1998). This difference between dairy and beef cattle has been observed in different abattoirs throughout Ecuador, and it was attributed to several factors such as cattle density, management practices and breed; however, data have never been published (personal communication). In Ecuadorian dairy industry, imported European breeds have been used to improve milk production, i.e. Holstein Friesian, which is less resistant to BTB than Zebu (Omer et al., 2001; Ameni et al., 2007; Kazwala et al., 2001).

Compared to the recently observed CITT-based prevalence data among dairy cattle from the Mejia canton (Proaño-Pérez et al., 2009), our veterinary-inspection-based prevalence was lower. This could reflect the lower sensitivity of post-mortem examination (Norby et al., 2004), implicating that a high number of animals might be classified as negative by this method. Routine abattoir inspection can be affected by the method employed, the anatomical sites examined and the stage of infection (Corner, 1994); it will fail to detect infected animals if lesions are present in tissues not being inspected. Since the veterinary inspection in our survey was done in a very comprehensive way, we believe that the early stage of infection played a role in the low detection rate. During our abattoir survey only one of the 5 bovine CITT reactors had evident gross lesions; the rest showed only minor lesions in thoracic lymph nodes. Three of the latter were identified as CITT-reactors in the year of slaughter, and therefore might be at the early stage of infection. The two remaining animals were CITT-reactors in two consecutive years and slaughtered afterwards; one of them showed characteristics of advanced disease. Nevertheless, BTB was confirmed by laboratory tests in all CITT-reactors. These findings confirm that animals only show evident characteristics in an advanced stage of the disease (Corner, 1994), although the symptoms of the disease are not pathognomonic. On the other hand, our data also confirm that *M. bovis* can be isolated from animals without gross lesions, including lungs (Whipple et al., 1996; Teklul et al., 2004).

Gross visible lesions may be caused by other organisms as well, and also NTM may constitute a confounding factor. In Tanzania, Cleaveland and colleagues (2007) identified *M. terrae*, *M. avium*, *M. chelonae*, *M. gordonae*, *M. fortuitum*, *M. flavescens* and *M. smegmatis* in samples from slaughtered cattle with visible lesions; however, the clinical relevance of the isolation of these environmental mycobacteria needs to be confirmed. In Ecuador, the presence of *M. avium-intracellulare-scrofulaceum*, *M. gordonae*, *M. szulgai* and *M.*

*celatum* was reported in slaughtered dairy cattle (Proaño-Pérez et al., 2006). Therefore, the identification of the species plays a crucial role in the final diagnosis of the disease. We did not isolate NTM in the present study.

The combined use of microscopy, *in vitro* culture, PCR and histopathology identified BTB in 51.5% (17/33) of the suspected animals. Nevertheless, only 6 of these animals were positive by all the laboratory methods used.

*M. bovis* was identified by *in vitro* culture in 36.34% of the suspected cattle. This is low compared to studies from Mexico and Argentina. In Mexico, BTB infection was confirmed by culture in 59% of cattle from 6 important dairy regions (Milian-Suazo et al., 2000), and in the Province of Santa Fé, Argentina, with 83% of samples showing visible lesions (Latini et al., 1997). The *Se* of detection by culture can be reduced due to the applied decontamination procedure or pre-analytical storage conditions of the samples, affecting the viability of the Mycobacteria (Palomino and Portaels, 1998). Freezing–thawing cycles proved to reduce the viability of mycobacteria significantly (Portaels et al., 1988). As our specimens underwent two freeze–thaw cycles, part of the bacilli may have been killed and therefore could not be isolated. AFB were detected in 14 of 94 specimens, but in 4 of them the presence of *M. bovis* could not be confirmed by culture. This might be attributed to the suboptimal storage conditions not ensuring survival of the bacteria. Nevertheless, our culture positivity rate was still higher than that observed in some other studies. In Brazil, culturing of *M. bovis* was successful in 18% of the samples (Pires de Araújo et al., 2005); no information was given on the storage conditions of the investigated samples.

The detection rate by PCR was even lower in our study. Applied on decontaminated tissue samples, it could detect BTB in 27.3% of suspected animals. In Brazil, 18% of cattle showing gross lesions yielded a positive PCR (Pires de Araújo et al., 2005). The *Se* of nucleic acid amplification can be influenced by different factors, such as insufficient quantity of bacilli or the presence of inhibitors hampering the polymerase (Mangiapan et al., 1996; Pires de Araújo et al., 2005; Cardoso et al., 2009). In Brazil, comparison between different concentrations and volumes of DNA samples showed that the percentage of PCR-positive lymph nodes increased from 39.4% to 54.5% after re-analysis of the initially negative samples using a 1:2 dilution of the suspension and 2.5 µl instead of 1 µl (Cardoso et al., 2009). In our study, we used 10 µl of undiluted DNA extract for the PCR, implicating that the sensitivity of our PCR might increase by the use of diluted DNA extracts, thus minimizing the effect of possible inhibitors.

We observed a good agreement between culture and PCR ( $\kappa = 0.61$ ), implicating that a high number of samples yielded a positive result by both tests, and confirming that culture and PCR are the most important diagnostic tools for the diagnosis of BTB in dairy areas (Cardoso et al., 2009).

Histopathology showed BTB compatible images in 27.3% of the cattle with macroscopic lesions. Demelash et al. (2009) showed that the *Se* of histopathology diagnosis can be affected by the technique used and the number of slides examined. In our study, two slides per specimen were examined, i.e., after HE and after ZN staining. On the other

hand, only 5 of the 9 animals identified by histopathology were positive by all laboratory tests.

The diagnostic accuracy of a test is primarily defined by its *Se* and *Sp*. However, these parameters can be influenced by minor changes occurring when processing samples. In this study, culture was considered the gold standard method to estimate the *Se* and *Sp* of the remaining diagnostic tests. It is important to consider that all samples were paucibacillary and pre-analytical sample conditions were suboptimal for culture because of the two freezing-thawing cycles. Therefore, the observed sensitivities of PCR (56.5%), microscopy and histopathology (43.5% each) could be overestimated. Microscopy is known to have a low *Se*, as it requires 5000 to 10,000 bacilli/mL of sample to yield a positive result. The *Se* of PCR and histopathology could be influenced by factors described above.

In routine conditions, it is not feasible to apply all these laboratory tests for all samples, given the high cost and labour required. Therefore, the best strategy should be chosen to confirm the suspected cases when implementing a BTB control program in Ecuador. The results obtained in this study suggest that PCR is a good alternative for culture, yielding results in a much shorter time. However, as it counts for culture, PCR needs to be performed by trained personal in an appropriate, quality-ensured laboratory. On the other hand, microscopy is rapid, easy, and cheap, and it can be implemented in resource-poor settings (Hirao et al., 2007). Because of its low sensitivity, all smear-negative samples should be retested by culture and/or PCR. A possible strategy to perform post-mortem diagnosis for BTB could be as follows: veterinary inspection, sampling of tissues showing gross visible lesions and lungs, smear microscopy to detect AFB, and PCR on all smear-negative samples.

Our second objective was to study and characterize the distribution of lesions observed in the organs from slaughtered cattle as a surrogate marker for the possible route of transmission and stage of disease (Phillips et al., 2003). During our survey, macroscopic lesions were found in mediastinal (51.32%), tracheobronchial (23.68%), hepatic (11.84%) and retropharyngeal lymph nodes (9.21%), and in other sites (3.95%). Mild lesions in lungs were found in only one slaughtered bovine. Similar observations were published in the United States and Iran, where no gross lesions suggestive of BTB were observed in lungs from dairy cattle, but frequently in lymph nodes of the thoracic region (Whipple et al., 1996; Tadayon et al., 2008). On the contrary, affection of lungs and other organs has been reported in cattle in Africa. In Ethiopia, 84% of visible lesions were found in the lungs and thoracic lymph nodes (Teklul et al., 2004). In Mali, 79% of the animals had confirmed lesions in lungs, and the infection with *M. bovis* was highly associated with these lesions ( $p < 0.001$ ) (Müller et al., 2008). However, in Tanzania, visible lesions were mainly found in the gastrointestinal tract (61.3%) (Cleaveland et al., 2007); these differences could be attributed to the breeds involved in the studies.

It is important to also consider that different *M. bovis* genotypes may differ in pathogenesis and thereby affect the development of lesions (Goodchild et al., 2003; Ameni et al., 2007; Oloya et al., 2007). In our study all ani-

mals with macroscopic lesions were Holstein Friesian and infected by the same strain (SB0980; Proaño-Pérez et al., in preparation). The observed inter-animal heterogeneity of macroscopic and microscopic lesions therefore most likely represents the individual diversity of pathological evolution of BTB (Meikle et al., 2007).

The macroscopic lesions found in dairy cattle slaughtered during this survey suggest that the most important route of transmission among the cattle studied was through respiratory tract.

## 5. Conclusion

In conclusion, our data demonstrate that veterinary inspection in combination with laboratory-based analyses is useful to document the presence of BTB, and they urge for the implementation of a national BTB control program. It should combine *in vivo* skin testing before slaughter to identify early infections with *M. bovis*, with extended post-mortem inspection including laboratory-based diagnosis. These activities should be performed by qualified veterinarians and technicians, following strict safety procedures to avoid human infections. Veterinary inspection needs to be implemented as a routine procedure in all abattoirs located in areas with a high cattle density. However, this can only be achieved if BTB is declared an important disease for animal and human health in Ecuador, and the required financial support is provided to support inspections, testing and compensation for losses.

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### **3.4 Single *Mycobacterium bovis* strain prevailing among dairy cattle in northern Ecuador**

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*Journal of Preventive and Veterinary Medicine* (submitted)

**Single *Mycobacterium bovis* strain prevailing among dairy cattle in northern Ecuador.**

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**Abstract**

Spoligotyping has been used for molecular identification of *Mycobacterium bovis* strains in Ecuadorian dairy cattle. From 1390 slaughtered cattle, it was possible to isolate 23 *M. bovis* strains with the same spoligotype named SB0980, according to the *M. bovis* molecular typing database. The homogeneity of the strains could be related to the relatively small study area; therefore it is necessary extent molecular epidemiology studies in all provinces of Ecuador.

**Key words:** Spoligotyping / *Mycobacterium bovis* / dairy cattle / Ecuador

**Introduction**

Even though national prevalence data remains unknown, isolated studies have shown that bovine tuberculosis (BTB) is still very prevalent among dairy cattle in Ecuador (Proaño-Pérez et al., 2006; Proaño-Pérez et al., 2009). There is no national BTB control program in place, because the disease has not been declared as an animal or public health importance. Consequently, molecular identification of prevailing *M. bovis* strains has not been performed, and thus data on *M. bovis* strain diversity are lacking in Ecuador. Spoligotyping is an important tool for molecular epidemiology studies on BTB (Müller et al., 2008; Milian-Suazo et al., 2008; Ameni et al., 2007; Asiimwe et al., 2009). This DNA-fingerprinting technique is based on the strain-dependent hybridization patterns of amplified DNA reacting with multiple spacer oligonucleotides specific for the direct repeat region of *M. tuberculosis*-complex (Kamerbeek et al., 1997).

The aim of this study was to type strains causing BTB among dairy cattle in the Mejia canton, the major dairy cattle production area in northern Ecuador.

### Materials and methods

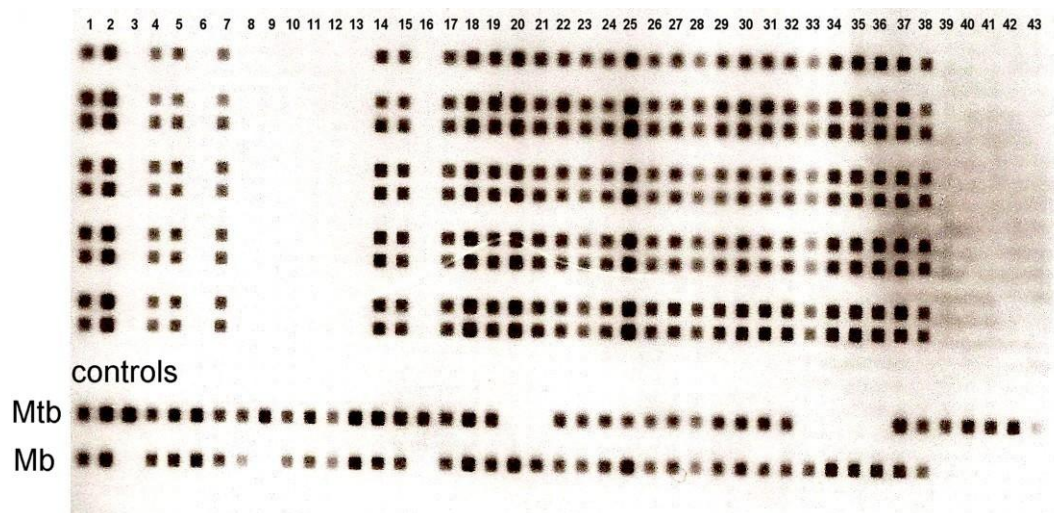
Veterinary inspection was carried out in 1390 slaughtered dairy cattle at the abattoir of Machachi located in the Mejia canton (manuscript for publication). Detailed postmortem examination was performed in 687 and 703 cattle, during the period from February to March in the years 2007 and 2008, respectively. The lungs, liver, spleen, kidney, mammary gland, mandibular, retropharyngeal, tracheobronchial, mediastinal, hepatic, mesenteric, and supramammary lymph nodes were inspected. Individual data (age, breed, sex, name of the owner, origin of the animal, and lesions found) were recorded for each animal. All cattle originating from areas outside the Mejia canton were excluded for the prevalence study. Samples were stored in a cool box, and transported the same day towards the Laboratory of Microbiology at the International Centre for Zoonoses, in Quito. Approximately 5 grams of tissue was stored at -20°C in 2mL Eppendorf® tubes containing Dubos broth (No. 0385-17-6, Difco Laboratories, Detroit, USA) supplemented with PANTA (Bactec PANTA plus kit No. 440 476 4; Becton Dickinson, New Jersey, USA) to inhibit the growth of contaminants and to preserve the mycobacteria. Samples were cut and homogenized in a sterile mortar with 2mL of sterile phosphate buffered saline. *In vitro* culture was carried out after decontamination using the reversed Petroff method (Portaels et al., 2001), and inoculation on Stonebrink media containing 0.5% sodium pyruvate.

Spoligotyping was conducted as described by Kamerbeek, et al. (1997). Identification of the obtained profiles was carried out by the use of the *M. bovis* molecular typing database ([www.mbovis.org](http://www.mbovis.org)).

### Results and discussion

Twenty-three isolates from 17 different animals were identified as *M. bovis*, typically lacking spacers 3, 9, 16 and 37 - 42 (Kamerbeek et al. 1997). All isolates showed the same spoligotype (Figure 1), named SB0980 according to the *M. bovis* molecular typing database. The strain was found in 7 different farms located in 5 districts of the canton (Figure 2) i.e., Machachi (2 farms), Aloasi (2 farms), Tambillo (1 farms), Aloag (1 farm) and Chaupi (1 farm).

**Figure 1. Spoligotype patterns of 9 *M. bovis* isolates from dairy cattle in the Mejia canton in Ecuador as compared to the *M. tuberculosis* H37Rv and *M. bovis* BCG reference strains.**



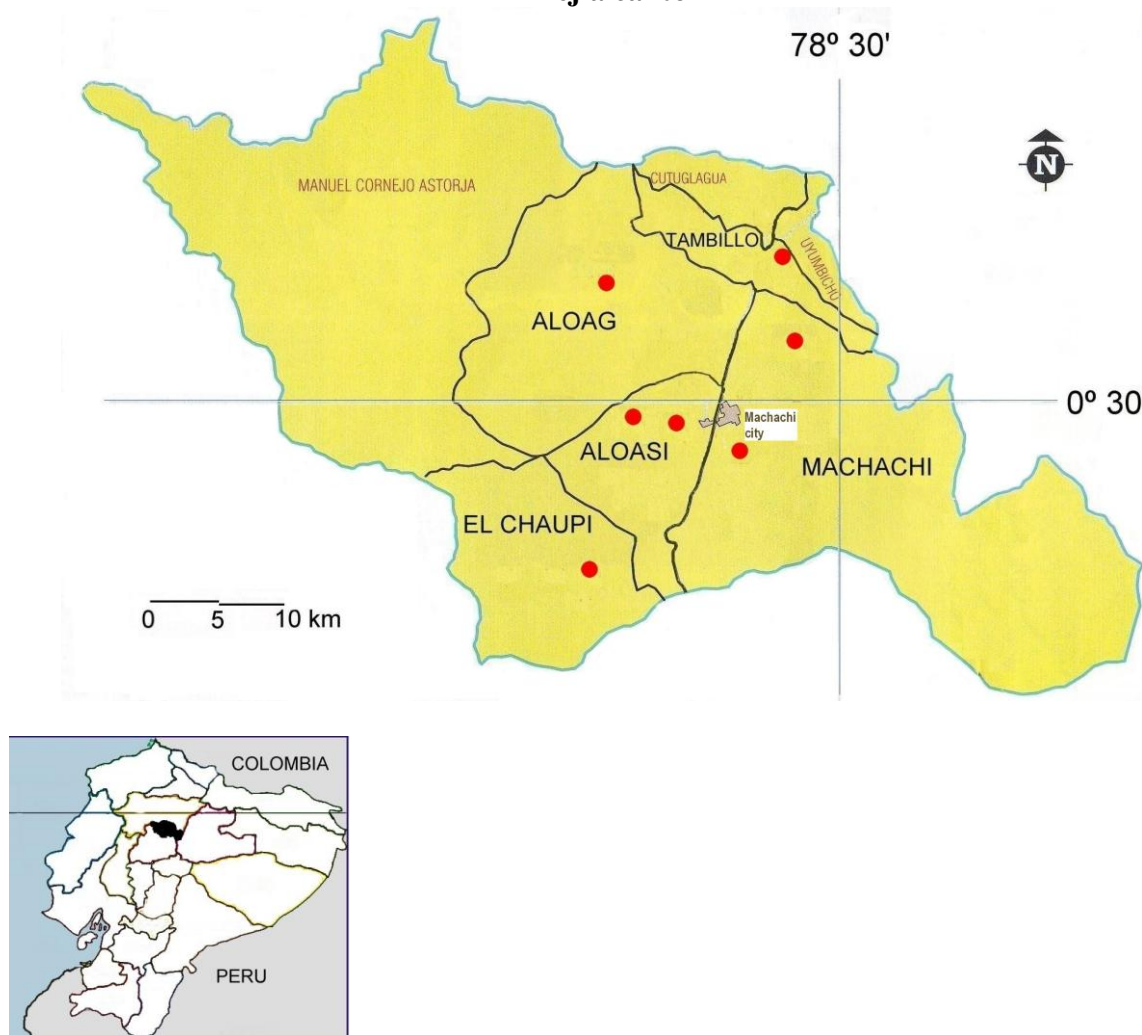
Mtb = *M. tuberculosis* H37Rv, Mb = *M. bovis* BCG

Lanes 1-9: *M. bovis* isolates from dairy cattle; Lane 10 Mtb = *M. tuberculosis* H37Rv; Lane 11 Mb = *M. bovis* BCG

The low polymorphism in our study could be related to the relatively small study area, a common source of infection for the studied cattle population, the fact that no new life cattle was imported in this region, and/or the absence of a wild life source infecting cattle. This finding of a single strain prevailing among cattle differs from data obtained in Brazil and Mexico, where a higher diversity was observed: 12 spoligotypes among 163 isolates from 2 states in Brazil, and 33 spoligotypes among 50 isolates obtained in 8 states from Mexico (Zanini et al., 2005; Milian-Suazo et al., 2008). The higher genetic diversity observed in Brazil and Mexico might be a consequence of the unrestricted movement of cattle between geographic areas within the country, and the fact that BTB is endemic in those countries (Milian-Suazo et al., 2008). As this is the first study of its kind in Ecuador, we can not confirm whether this clonal spread is representative for the entire country or whether different or more spoligotypes are present in the rest of the country.



**Figure 2. Distribution of *M. bovis* spoligotype SB0980 isolated from dairy cattle in the Mejia canton**



Nevertheless, this data contribute to understand the origin of the disease in this dairy region. This particular strain has been reported in the Republic of Ireland ([www.mbovis.org](http://www.mbovis.org)) and in Texas, USA (Milian-Suazo et al., 2008). It is very similar to spoligotype SB0140 carrying spacer 13 in addition, and found in cattle from the UK, Republic of Ireland, France, Australia, Argentina, Venezuela, Mexico, and Iran (Cousins et al., 1998, Tadayon et al., 2008). This data suggest that most probably *M. bovis* was introduced along with the importation of European breeds, mainly Holstein Friesian cattle in the middle of the 20<sup>th</sup> century, when the dairy sector started to develop in Ecuador. *M. bovis* spoligotypes have been reported to change *in vitro* in a timescale of approximately 60 years (Frang et al., 1998). Thus, the homogeneity of isolates found in our study reflects a short timeframe of events, suggesting that BTB appeared here from after the 1950s onwards. Although our spoligotype differs from

## Study 4

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all 12 spoligotypes found in the South-east of Brazil, it has also been suggested that BTB was introduced independently in that country along with cattle imported from Europe (Zanini et al., 2005).

Nowadays, around 90% of dairy cattle in the Mejia canton are Holstein Frisian, and it is known that European breeds are less resistant to BTB than Zebu (Omer et al., 2001; Ameni et al., 2007). Our observations coupled with the fact that movement of cattle between farms is very limited, and no Zebu cattle were found positive - even in farms with positive Holstein Frisian cattle- support this data.

### Conclusion

As only one *M. bovis* spoligotype was identified, specific routes of intra-cattle transmission could not be documented in this study area and period. Continued surveillance of BTB among cattle in all provinces of Ecuador, supported by spoligotyping data to properly identify the causative agent and type individual strains, is needed to confirm our findings and to document possible geographical and chronological heterogeneity.

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### **3.5 Risk analysis for infection of *Mycobacterium* spp. in a human population in Ecuador**

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In preparation

## Risk analysis for infection of *Mycobacterium* spp. in a human population in Ecuador.

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### Abstract

Tuberculosis (TB) in humans is mainly caused by *Mycobacterium tuberculosis*. However, the pathogenic agent of bovine TB, *Mycobacterium bovis*, can also cause the disease in human beings (zoonotic TB). This study was carried out in 157 people, conformed by farm workers and slaughterhouse workers from Mejia canton, considered an important dairy area in Ecuador located in Pichincha Province. The purpose of this research was to evaluate the prevalence of *Mycobacterium* spp. in risk populations by tuberculin skin test (TST) and to analyze the risk factors associated with the transmission of the disease. The results establish a significant association among the positivity of TST to masculine gender ( $P=0,026$ ), consumption of milk coming from farms ( $P=0,030$ ), consuming raw milk ( $P=0,000$ ), and consumption of elaborated cheeses made by hand ( $P=0,003$ ). Consumption of raw milk gave an *odds ratio* of 4,65 [ C.I.= 2,01-10,75 at 95% ]. The prevalence of *Mycobacterium* spp. was estimated in 29% with [C.I. = 22-36% at 95%] for this population.

**Key words:** bovine tuberculosis, Ecuador, *Mycobacterium bovis*, purified protein derivative (PPD), zoonoses.

### Introduction

Although *Mycobacterium tuberculosis* is mainly the causal agent of human tuberculosis (TB), *Mycobacterium bovis*, the etiologic agent of bovine tuberculosis, can also be responsible for the disease in human beings, which makes these bacteria an important zoonotic species (Cosivi *et al.*, 1998, Taylor *et al.*, 2007). Current taxonomy recognizes 8 members inside the *Mycobacterium tuberculosis* Complex: *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. caprae*, *M. africanum*, *M. pinnipedii*, *M. microti* and *M. cannettii* (van Soolingen *et al.*, 1997; Cousins

Study 5

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*et al.*, 2003; Aranaz *et al.*, 2003; Mostowy *et al.*, 2005; Pavlik *et al.*, 2005; Taylor *et al.*, 2007).

Success of the tubercle bacilli as a pathogen is due to its ability to persist in the host during long periods of time and to cause the disease overcoming the immune response of the host (Flynn and Chan, 2001). Tubercle bacillus survives well in not pasteurized milk and its sub products. It has been reported that dairy products like yogurt and cheese, manufactured from milk coming from infected animals can store the bacillus more than 14 days after the elaboration of these products and in butter can remain contaminated more than 100 days (Kleeberg (1984) mentioned by Gallagher and Jenkins, 1998).

TB caused by *M. tuberculosis* or *M. bovis* is clinically, radiologic and pathologically indistinguishable (Wedlock *et al.*, 2002). Human-human infection with *M. bovis* is usually considered as a rare event, however the high prevalence of HIV combined with the presence of several risk factors could change the epidemiology of the disease in the human guest human-human transmission and be more common (Ayele *et al.*, 2004).

Human infection with *M. bovis* can occur by inhalation of droplets coming from infected animals as the result of the animal's excretion but they can also be produced during the handling of slaughtered animals with tuberculous lesions (Neill *et al.*, 1989); another possibility for infection is the ingestion of not pasteurized milk coming from sick animals. Thereby, people that work in animal husbandry, slaughterhouse workers, veterinarians and people in close contact with possible infected animals are those that could be in a higher risk to acquire *M. bovis* infection (Cousins and Dawson, 1999).

Immune response against mycobacteria infection is mainly cellular, the delayed type hypersensitivity reaction (DTH) stimulated by the antigens present in the bacteria, traditionally has been quantified by the TST (Reece *et al.*, 2005). This test has turned out to be an advantageous method and low cost to determinate the cellular immune response toward the purified protein derivative (PPD) obtained from mycobacteria (Reichman, 1976; Katial *et al.*, 2001). Identification of infected people with the tuberculous bacillus, especially in epidemic studies has been carried out by this test (Reece *et al.*, 2005).

## Study 5

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In Ecuador as well as in other countries of the region, the rate of human incidence of TB has regularized and it even has begun to descend lightly in the last years (WHO, 2007), as a result of the implementation and consolidation of DOTS strategy (Directly Observed Treatment Short Course) especially in provinces with a higher human density (MSP, 2007). In 2005, the Ministry of Public Health (MSP) reported 3854 cases of pulmonary TB with a positive baciloscopia (BK+), 401 cases of extra-pulmonary TB and 1259 deaths caused by TB at a national level (MSP, 2006). Studies concerning human TB caused by *M. bovis* do not exist in Ecuador; the Ministry of Public Health, Ministry of Agriculture or the Ecuadorian Service of Animal Sanitary (SESA) have never notified any case.

The objective of this study was to identify *Mycobacterium* spp. in human populations at risk in Mejia canton in Ecuador through immunologic test (TST) and determine its prevalence; in addition, a survey was applied to evaluate the risk factors involved in the possible transmission of the disease among humans.

## Materials and Methods

### Study population

The study group was formed by 157 people belonging to 9 farms (n=92) and a slaughterhouse (n=65) from Mejia canton in Pichincha province located in the north region of Ecuador. To select the participants to form this group were taking into account the followings aspects: 1) Patients under 15 years were excluded of the study to avoid confusions caused by the vaccination with BCG. 2) Patients do not report chronic diseases as hypertension, diabetes, HIV/AIDS, recent history of vaccination against flu and smallpox and immunosuppressor treatments.

### Tuberculin skin test (TST)

After the agreement of each patient, TST was performed administrating the antigen (PPD) injected intradermally in the middle half surface of the right forearm, 2 IU of PPD RT-23 (Statens Serum Institute, Copenhagen, Denmark, lot n° 685461.7), equivalent to 5 IU of PPD-S contained in 0,1 mL of solution. The test was considered successful when it was observed an elevation of at least 6 to 10 mm occurred in the skin after the injection.

## Study 5

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Measuring was done 72 hours after inoculation through this procedure: first, palpate the area to determine the margins of induration to differentiate it with edema and define the borders through the ballpoint method described by Sokal (1975). The margins of skin induration were determined based on the trace a perpendicular line to the axis of the forearm, from a distant point 1-2 cm outside of the margins of the induration and then directed to the center of it (ATS/CDC, 2000). The result of the reaction was registered in mm of induration, positive reaction was defined as a reaction  $\geq 10$  mm, recommended for populations where TB is endemic (ATS/CDC, 2000), and it is the traditional cut-off used with PPD RT-23 in most of these studies in the world (Wang *et al.*, 2002).

### Survey applied

A Complementarily survey (n=124) was carried out to determine the possible risk factors involved in the exposure for TB, the questionnaire was structured in four aspects: housing conditions, symptoms of the disease, milk and cheese consumption habits and contact with bovine livestock. The presence of symptoms related to pulmonary TB and TB extra pulmonary were analyzed, as well as cultural aspects of dairy products consumption and especially giving more attention in the consumption of not pasteurized products and protection measures when people work with animals.

### Statistical analysis

The estimated prevalence was calculated with the positive results of TST, with 95% of confidence intervals (C.I) and normal distribution of the estimator. The characteristics of positive reactors by TST vs. the non-reactors were compared using the statistical association Chi-square for two nominal variables ( $\chi^2$  of Pearson, G Test, Exact Test of Fisher) and with association measures (Contingency Coefficient, Index of correlation of Spearman) by the use of contingency tables. Additionally, in those cases with significant or highly significant differences, comparison of proportion reasons *odds ratio* (OD) was carried out and allowed to measure association of risk factor in relation to its occurrence (Agresti, 1996). Multiple correspondence analysis (MCA) was carried out to correlate variables that showed significant differences using the juxtaposition of several contingency tables (Sánchez, 2006). All the statistical analyses were interpreted at two tails to estimate the p value at 95% of C.I. and the data analysis was applied with a statistical package SPSS® version 13.0 for Windows.

## Study 5

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### Results

#### Study population

Total studied population (n=157) was distributed in 58,6 % farm workers and 41,4% slaughterhouse employees, with an average age of 37,6 years old (y) [minimum 15y; maximum 79y] standard deviation (SD):  $\pm 13,1y$ . The mean time working with animals was 10,4y with a SD:  $\pm 10,8y$ . 42,0 % of people inhabited in Machachi city, 27,0 % lived in the farms and 31,0% came from towns near to their working place. Table 1 shows the distribution of the study population where the TST was carried out.

#### Survey prior the TST

Of the 157 any patient manifested to have been diagnosed with TB as well as neither to have been related and/or to know cases of people with active TB and 97,5% mentioned they had never went through TST previously, 145 patients (92,4%) had been vaccinated with BCG, and from this percentage 55,8% received a second vaccine as reinforcement. Likewise, 33 patients (21,0%) had undergone to HIV/AIDS test, all of them with a negative result.

#### Tuberculin Skin Test (TST)

From the total 157 patients tested by TST, 152 (96,8%) returned after 72 hours for the reading of the reaction in the skin, the average reaction was 5,3 mm with a SD:  $\pm 6,1$  mm. The results of TST revealed 108 cases (71,1%) with a negative reaction ( $< 10$  mm) and 44 cases with a positive reaction ( $\geq 10$  mm), meaning an estimated prevalence of 29,0% with C.I. = [22,0-36,0]. Inside of the positive reactors group the average age was 40,46y with a SD:  $\pm 13,05y$  and the average size of the reaction to the tuberculin was 13,80 mm with a SD:  $\pm 3,012$  mm.

#### Survey

In 124 patients (79,0 %) a survey related to the risk factors was applied and these data were analyzed using the statistical analyses of association.

#### Related symptoms

11,9% of the study group manifested to have frequent cough and 11,1% to have presented cough at some time during more than 15 days. With regard to other symptoms related to the TB it was found that 15,0% manifested night perspiration, 15,9% loss of weight, 17,5% stomach pains, 3,2% frequent diarrheas; 23,0% renal pains and 8,7% urination malaise.

## Study 5

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### ***Consumption habits of dairy products and bio-security***

88,9 % of the patients consume milk, from this percentage 34,8 % had consumed or they are still consuming raw milk, while 65,2% manifested that they do not ingest raw milk or have always boiled it. The precedence of the milk is 40,2% from farm properties directly, 10,7% of consumed milk came from small supermarkets in that area, 16,1% consume milk coming from their own cows and 37,0% declared to only buy milk in small supermarkets, being assumed that this milk was subjected to the pasteurization process. In respect to the consumption of cheese we found that 7,1% did not consume this product and, on the contrary, the great majority (92,9%) did. We found that the provenance of cheese for consumption mainly came from artisans (47,0%) as well as of stores (47,0%) and 6,0% manufactures their own cheese and in this case, 89,7% declared to use raw milk for the elaboration of the cheese while 10,3% only used pasteurized milk for its elaboration. In relation with the biosafety measures 5,6% manifested to use a mask when they work with livestock and 42% to use gloves.

### **Association analysis among variables**

Several contingency tables were performed to determine if some relationship existed among the different variables in study. There was not significant association ( $p > 0,05$ ) among TST results and patients' age, kind of work, working time and BCG vaccination (Table 2). However, it was found a significant association ( $p = 0,026$ ) among gender (masculine or feminine) and positive or negative reaction to TST, with a degree of correlation among both variables also significant ( $p = 0,021$ ) which was given by a value of Spearman correlation of -0,187 which indicates that, according to the order of the categories, the fact of being woman is associated with a smaller number of positive results, the *odds ratio* for this risk factor among both variables was 2,52 with C.I. = [5,61-1,131] what indicates that between the comparison of both genders men have more than double probability to be positive by TST regarding with respect to women in this population.

**Table 1. Results of the independence analysis by the use of contingency tables**

Contingency Table	$\chi^2$ of Pearson	G Test	Fisher Test (n-tails)	P value
<b>1. Housing conditions</b>				
TST * Group Age	-	2,428	-	0,488 <sup>N.S.</sup>
TST * Occupational area	4,248	-	-	0,373 <sup>N.S.</sup>
TST * Working time	-	7,010	-	0,072 <sup>N.S.</sup>
TST * BCG	-	-	x (2)	0,330 <sup>N.S.</sup>
TST * Gender	-	-	x (2)	0,026*
<b>2. Symptomatology</b>				
TST * Frequent cough	-	-	x (2)	0,773 <sup>N.S.</sup>
TST * Cough more 15 days	-	0,007	-	0,931 <sup>N.S.</sup>
TST * Cough with phlegm	-	-	x (2)	0,101 <sup>N.S.</sup>
TST * Night Sweating	-	-	x (2)	0,600 <sup>N.S.</sup>
TST * Stomach pain	-	-	x (2)	0,137 <sup>N.S.</sup>
TST * Stomach scratches	-	-	x (2)	0,274 <sup>N.S.</sup>
TST * Ardor when urinating	-	-	x (2)	0,512 <sup>N.S.</sup>
TST * Renal pain	-	-	x (2)	0,071 <sup>N.S.</sup>
<b>3. Milky habits consumption</b>				
TST * Consumption of milk	-	-	x (2)	0,769 <sup>N.S.</sup>
TST * Type of milk	-	-	x (2)	0,000**
TST * Provenance of the milk	7,025	-	-	0,030*
TST * Consumption of cheese	-	0,030	-	0,863 <sup>N.S.</sup>
TST * Provenance of the cheese	-	11,562	-	0,003**
TST * Production of cheese	-	-	x (2)	0,044*
TST * Type of milk frabr. cheese	-	-	x (2)	0,224 <sup>N.S.</sup>
<b>4. Biosafety</b>				
TST * Near contact with animals	-	-	x (2)	0,556 <sup>N.S.</sup>
TST * Basic protection	-	-	x (2)	0,140 <sup>N.S.</sup>
NS not significant association      * significant association      ** highly significant association				

Results obtained from the risk factors using the survey determined that there was not significant association ( $p > 0,05$ ) among the positive or negative result of the TST in relation with age, occupational area, working time, BCG vaccination, symptoms related with pulmonary or extra pulmonary TB, intestinal or genito-urinary illness, consumption of milk or cheese, close contact with animals and basis protection (Table 2).

However, among consumers of milk we found a highly significant statistical differences ( $p < 0,01$ ) among patients that consume raw milk versus boiled milk in relation to the TST result. Association degree among both variables was also highly significant ( $p < 0,01$ ) and it was given by the correlation value of -0,352 which demonstrates that according to the order of categories in the contingency table, consuming boiled milk is associated with a smaller



## Study 5

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number of positive results to the TST. The *odds ratio* value of risk relationship among both variables was established in 4,65 with C.I. = [2,01-10,75], people who drink raw milk have more than four times the probability to be positive by TST related with those drink boiled milk.

The provenance of the milk had significant differences ( $p=0,030$ ) with the TST result, the association degree among both variables was significant ( $p=0,030$ ) and it was given by a contingency coefficient of 0,245, meaning that if the milk is less treated (pasteurization) a bigger number of positive cases will be found in the TST (Table 2).

A highly significant association ( $p<0,01$ ) was found among the provenance of cheese and TST result, the association degree showed significant differences ( $p=0,018$ ) and value of Spearman correlation of -0,218 what it means that better quality of milk to make cheese represents a higher number of negative cases. Likewise, there were significant differences ( $p=0,044$ ) among people who manufacture cheese and those that do not make it with the TST result, the association degree among both variables was significant ( $p=0,028$ ) obtaining a value of Spearman correlation of 0,198 which indicates that manufacturing cheese is associated with a bigger number of positive results (Table 2).

### Multiple Correspondences Analysis

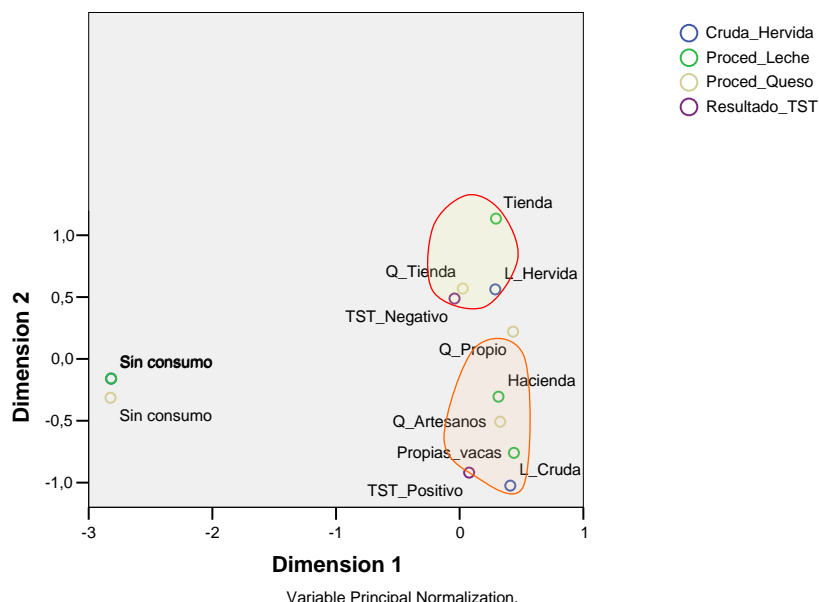
The MCA or multiple correspondences analysis allowed us to compare more than two variables of nominal response. Four contingency tables were performed using the results with significant differences to independence analyses in relation to TST result analyzed with: consumption of raw or boiled milk, provenance of milk and cheese. We fulfilled the condition that the rows of all the tables correspond to the same individuals and the columns to different variables with their respective classifications. Figure 1 allows identify the relationship between the variables, it was observed that patients with positive result ( $\geq 10$  mm) mainly were related with consumption of raw milk, consumption of milk coming from farms, consuming milk from their own cows and buy handmade cheeses.

In contrast, patients that had a negative result to the TST ( $< 10$  mm) were related with the consumption of boiled milk, milk and cheese bought it in small supermarkets and when they

## Study 5

manufacture them homemade. Finally, the group of people that doesn't consume milk and/or cheese didn't have any relationship with the analyzed variables.

**Figure 1. Quantification Plot of the categories using Multiple Correspondences Analysis**



## Discussion

The results obtained in this study demonstrate that the analyzed population is in risk; from 157 farm and slaughterhouse workers, 44 were positive reactors to tuberculin skin test representing almost one third of the study population (29,0%), these findings were correlated with the information obtained by the use of a survey. In Ecuador an important sub-registration of TB is calculated as a consequence of the detection and report of the pulmonary symptomatic cases exclusively. This situation has generated that the Control Program for Tuberculosis of the PHO/WHO considers Ecuador like a country of high prevalence/incidence, with rates 2 to 3 times superiors to those reported at a National level and situate Ecuador among the 10 countries with higher load of TB (MSP, 2006). Additionally, the number of human cases generated by *M. bovis* and which is the importance of this pathogen in our context is unknown.

## Study 5

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### **Tuberculin Skin Test (TST)**

This is the first study in Ecuador that investigated the relationship among positivity to TST and exposure to *Mycobacterium* spp. in a risk human population exposed to bovine infected cattle and their dairy products. It was used a reference cut off of 10 mm, which is recommended for populations in risk to determinate the positive cases for tuberculin reaction and estimate the prevalence infection with pathogenic mycobacteria (ATS/CDC, 2000). Although the data don't suggest that close physical contact with bovines is a risk factor to have a positive result by TST, the estimated high percentage of positive cases found in farm and slaughterhouse workers could reflect: (1) possible previous exposure to tuberculous animals (2) transmission of *M. bovis* from livestock toward the humans or (3) simply, previous contact with human TB cases (*M. tuberculosis*); in contrast to this aspect, all the interviewed did not manifested to have been related or did not know cases of people with active TB.

The significant statistical differences found in relation to the gender with a higher probability that men have a positive reaction to the tuberculin can be related according to Cousins & Dawson (1999), because men are more exposed to mycobacteria in one or another way when they work with livestock, representing a bigger occupational risk.

Reliability of TST can be a controversial thematic to discuss, false positive and negative results can be caused by intrinsic variability of this test such as dosage variations in the tuberculin, administration technique, lecture variability and anergy (Norval *et al.*, 2004). In order to avoid vials by these remarks it was used an uniform protocol based on rigorous administration of equals quantity of antigen (0,1 mL), maintenance of cold chain to avoid the degradation of the tuberculin, identical technique for lecture, interpretation and this procedure must be carried out by the same person. A couple of studies report that 4,0% of the reactor cases may be the probability of cross reactions generated by non tuberculous mycobacteria (NTM) reported by Rupp *et al.* (1994) and Grabau *et al.* (1995).

### **Risk factors analysis**

It is very difficult to determine with accuracy the transmission routes involved in the exposure to *M. bovis*, mainly due to the association of this pathogen with ingestion of infected milk or dairy products, as well as with the infection by respiratory route at the moment of breathing

## Study 5

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contaminated aerosols (LoBue *et al.*, 2004). The results previously presented show that there was not correlation between a positive TST and symptoms related with pulmonary disease (air transmission), neither with symptoms related to extra pulmonary disease which mostly could be associated with consumption of milk and cheese coming from animals with BTB.

Possible transmission of *M. bovis* from livestock toward humans has been reported in other studies, consumption of not pasteurized milk coming from bovines with TB is a risk factor involved in the infection (Winthrop *et al.*, 2005; Remacha *et al.*, 2006). The findings in this study suggest a high correspondence among consumption of milk coming from farms, raw milk and handmade cheese associated with a positive TST, on the contrary a negative TST is associated with consumption of boiled milk coming from the farms and with consumption of pasteurized milk and cheese from formal trades. In consequence, consumption of not pasteurized milk and their dairy products would constitute a risk factor for the infection and transmission of the zoonotic TB and probably contributed to the positive reactions observed in the TST.

In Ecuador there is not information about the distribution, epidemic patterns and dynamics of transmission of this important zoonosis and whose pathogen agent is *M. bovis*. In a study carried out by Proaño-Pérez *et al.* (2006) an estimated prevalence of 3,85% for BTB in Mejía canton was reported, this data confirms the high risk that exists for the transmission of the bacteria (air borne/or digestive) among sick animals with or without symptoms toward workers in close contact with them, this study did not show statistical differences in relation to this aspect. Nevertheless other studies have demonstrated this possibility as an inducible factor to development the disease in humans (Ameni *et al.*, 2003; Biet *et al.*, 2005; Evans *et al.*, 2007) especially when they have an attenuation of the immune system which can appear frequently due to deficiencies in health and nutrition conditions most commonly found in rural inhabitants.

In consequence, the main problem remains in the cultural habits from farm workers consuming raw milk that comes from sick animals, could be infecting themselves without knowing it and if by any chance they become immunosuppressed, the bacteria inside their bodies (latent infection) could pass to an active state and cause disease. On the other hand, the milk coming from the livestock with TB can be sold by individual people or artisans that own

## Study 5

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small factories to elaborate cheese and which do not complete the pasteurization process, and the pathogen can be distributed among consumers, making viable the infection with *M. bovis* with can survive more than 14 days in cheese and more than 100 days in butter (Kleeberg (1984) mentioned by Gallagher and Jenkins, 1998).

In slaughterhouse workers the survey demonstrated that they have different consumption habits of dairy products in relation to farm workers due to easy access that slaughterhouse workers have to shops and supermarkets inside the trade in the Machachi city, situation that was not presented in farm workers. This would be the reason to justify those people could be able to acquire pasteurized dairy products. Wilkins *et al.* (2003) have reported that the risk of *M. bovis* infection in slaughterhouse workers is given by the lack of hygiene inside the buildings, informal market that exists, absence of veterinary inspection, illiteracy of the disease and deficient use of gloves, all these aspects can facilitate the cutaneous infection particularly when workers cut themselves with knives during slice in sick animals.

The findings support the necessity to evaluate and quantify the impacts of the zoonotic TB in order to be able to outline to the sanitary services and different departments that are responsible of human and animal health to develop a control program especially in areas where the presence of the disease is significant. In Ecuador the lack of political will, the existence of an informal trade which does not fulfill appropriate sanitary rules and permits informal sell of dairy products without any control is a big problem, although the big dairy companies pasteurize most of the national production there is still a percentage that evades this process, this study showed 47% of the people buy fresh handmade cheese elaborated with raw milk at the.

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**3.6 Review article: Situation of bovine tuberculosis in Ecuador**

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**Temas de actualidad / Current topics****Situation of  
bovine tuberculosis in  
Ecuador**

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**SYNOPSIS**

Bovine tuberculosis (BTB) is a chronic and contagious disease that affects domestic animals, wildlife, and humans. Caused by *Mycobacterium bovis*, BTB causes major economic losses and poses a serious constraint to the international trade of animal products. Moreover, in developing countries where BTB controls are lacking, *M. bovis* is a public health concern. In most developing countries, the prevalence of BTB in livestock is unknown because the information is either not reported or not available. In Ecuador, there is no national BTB control program.

This article reviews the BTB situation in different geographic areas of Ecuador by examining exhaustive data reflecting tuberculin testing surveys, as well as slaughterhouse surveillance studies, from 1972 – 2008. In Ecuador, the dairy industry's expansion (pre-empted by the high demand for milk and its byproducts), intensified efforts to increase the cattle population, the presence of *M. bovis*, and a lack of BTB controls has caused a rise in BTB prevalence, and subsequently a growing push for the implementation of a national BTB control program.

**Key words:** *Mycobacterium bovis*; zoonoses; cattle diseases; tuberculosis, bovine; communicable diseases, emerging; Ecuador.

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Bovine tuberculosis (BTB) is a chronic infectious disease caused by *Mycobacterium bovis*. This disease mainly affects cattle, but can also be found in other domestic and wild animals, and occasionally, in humans (1). The World Organization for Animal Health (OIE) considers BTB to be an important zoonotic disease with a socioeconomic and public health impact that affects the international trade of livestock and animal products (2).

The prevalence of BTB in developing countries remains largely unknown. According to a study conducted in 2006, Ecuador is among a group of Latin American countries assumed to have a relatively high prevalence of BTB, but a lack of reporting (3). Other countries in the group are Argentina, Bolivia, Brazil, Chile, Guatemala, Guyana, Haiti, and Peru. In 1998, it was estimated that for 24% of the Latin American bovine population, control measures for BTB were incomplete or inexistent (4). This absence of control represents a high risk for the rural inhabitants living in direct contact with the animals (5). Of approximately 374 million cattle in Latin America and the Caribbean, 70% are held in areas where *M. bovis* infection rates among cattle exceed 1% (3).

In humans, the proportion of tuberculosis induced by *M. bovis* is relatively low compared to *M. tuberculosis*. But in recent years, *M. bovis* tuberculosis in humans has become increasingly prevalent where human populations are confronted with poverty, malnutrition, human immunodeficiency virus (HIV), and reduced access to health care (6). Transmission through the consumption of unpasteurized milk and dairy products from infected cattle occurs mostly among the general public, whereas exposure through airborne infection remains highest among farmers, veterinarians, and slaughterhouse workers (6). According to study published in 1998, *M. bovis* could be responsible for more than 2 % of the total pulmonary tuberculosis (TB) cases and 9.4 % of extrapulmonary forms among humans in Latin America, a considerable number. In addition, another study showed that in Argentina, 2 % of human TB cases have been recorded as being caused by *M. bovis* (3).

Given the appearance of new cases in recent years, BTB in humans has been designated a reemerging disease in developed countries. This reemergence is most likely the result of an increase in world population augmented by the movement of people and animals, plus environmental changes, the crossing of the interspecies barrier, and changes in the management of livestock production (4, 7, 8).

The total economic losses due to BTB are underestimated because its impact on public health has not been thoroughly evaluated (9). Estimates of economic loss are limited to animal health issues and are based on weight loss, decreased milk production, lowering of the reproductive rate, mortality, and condemnation of carcasses (10). In Argentina, the National Health and Food Safety Service (Servicio Nacional de Sanidad y Calidad Agroalimentaria - SENASA) estimates that in 1995 the annual losses due to BTB were approximately US\$ 63 million (4).

This article aims to provide information on the current situation of BTB in Ecuador based on available data from studies applying tuberculin skin-testing at farms, veterinary inspections at slaughterhouses, and laboratory-based diagnosis. The data presented in this article were obtained from the college libraries serving schools of veterinary medicine throughout the country. Most of the surveys were a component of veterinary students' theses. In Ecuador, there is only one group of researchers working on BTB, which explains the limited number of published studies.

## **BTB PREVALENCE**

BTB cases in Ecuador are not documented nor quantified clearly for several reasons: lack of proper recording of positive cases, limited use of diagnostic tests, and insufficient veterinary inspection in most slaughterhouses, but mainly because BTB is not a notifiable disease. Furthermore, it is not unlikely that in Ecuador—as may be the case in other countries—BTB is not reported due to a lack of trust between farmers and health officials (5). Currently, control measures related to relocation and transportation of animals focus solely on Foot and Mouth Disease. Furthermore, results of veterinary inspection are not recorded properly in slaughterhouses, jeopardizing the “traceability” of positive BTB cases observed during veterinary inspection.

The prevalence of BTB can be influenced by several factors at the individual, herd, and/or provincial/country level (11). For instance, livestock husbandry varies by herd size, farm size, and type of cattle industry, i.e., for dairy or meat production. Factors such as these can affect the occurrence of BTB disease by increasing or decreasing close contact between animals. In Ecuador, the cattle population is not equally distributed throughout the country:

## Study 6

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the provinces of Azuay and Pichincha have dense livestock populations predominantly focused on dairy production, while Santo Domingo and Manabí provinces have important meat industries (12), this distribution influence on the occurrence of BTB. The scarce information that is available in Ecuador is mostly based on tuberculin skin-testing performed by students at the schools of veterinary medicine within the various universities of Ecuador (Figure 1).

### **Tuberculin skin test surveys**

The sensitivity of skin testing can be affected by the potency and dose of tuberculin administered, the post-infection interval, desensitization, deliberate interference, postpartum immunosuppression, variation in the interpretation by the observer, and contact with environmental non-tuberculous mycobacteria (NTM) (13).

Surveys performed in Ecuador using the single and comparative intradermal tuberculin test (SITT and CITT, respectively) have demonstrated a variable prevalence of BTB. Briefly, the tuberculin skin test is based on the use of bovine purified protein derivative AN5 strain (PPD-B) 20 000 IU/mL and avian purified protein derivative D4 ER strain (PPD-A) 25 000 IU/mL, the test can be applied in the caudal fold of the tail (SITT) or in the middle third of the neck (CITT); the thicknesses of the skin is measured with calipers 72 hours before and after the injection of 0.1 mL PPD-A and PPD-B antigens. The interpretation is based on the reaction observed (14, 15). In most of the studies performed in Ecuador, the PPD antigens used were not reported.

Table 1 presents an overview of the tuberculin-testing surveys conducted in dairy cattle in Ecuador (16 – 21). In 1977, the first survey detected a low (0.3%) prevalence among dairy cattle in the Tungurahua province located in the middle of the country (16). A second study conducted in 2003 in the same area showed a 1.2% increase in the disease (17). In both studies, SITT was applied in all animals and CITT was used to confirm all positive and suspected cases detected. Data on the herd size and origin of the PPD reagents were not presented in these studies.



In contrast, in the province of Imbabura, a survey conducted in 2001 showed a high prevalence of BTB (7.3%) (18). However, 2 years later, it showed a lower prevalence (2.4%) (19). In the province of Carchi, located north of the Imbabura province at the border with Colombia, the prevalence observed in 2001 (2.1%) was comparable to that of 2002 (1.7%) (18, 20). In general, the prevalence found in the latter two provinces was higher than that of Tungurahua. This was probably due to the smaller cattle population and lower animal density of Tungurahua. Plus, Tungurahua is an area more focused on agriculture than animal production (12).

The majority of the surveys, however, were performed in the province of Pichincha, specifically in the cantons of Mejia and Cayambe, the two most important dairy areas, located near the capital city, Quito. In 2001, a prevalence of 2.8% was observed in the Cayambe canton (18). This finding was in sharp contrast to a prevalence of 0.5% reported a year later (21). Worth noting is that the first study sampled only two farms; therefore, the BTB prevalence data is questionable.

Studies performed in the Mejia canton showed a high occurrence of the disease in dairy cattle. In 2002, a study (22) observed 4.9% of skin reactors, which was confirmed by the prevalence of 3.9% found by another survey applied in the same area, but to different herds (14). The two studies used similar diagnostic methods, but sampling procedures differed. In the latter study, 22 cattle were randomly selected per herd from farms that were small (1 – 25 cattle), medium (25 – 70 cattle), and large (> 70 cattle). The diagnostic test used was CZV Bovine Tuberculin PPD<sup>®</sup> (CZ Veterinaria S.A., Porriño, Spain). The difference found related to herd size was highly significant: 7.9% in large herds; 3.4% in the medium; and surprisingly, only 0.3% in small herds (14). Recent information from CITT analysis, using Bovituber<sup>®</sup> PPD (Synbiotics Corporation, a subsidiary of Pfizer Incorporated, Lyon-France), of 20 dairy herds in the Mejia canton showed an annual incidence of 1.7%, with a true prevalence at 7.1%. Herd size was identified as a main risk, as were age ( $P = 0.03$ ), contact with other animal species ( $P < 0.01$ ), and introducing new animals into the herd ( $P = 0.04$ ) (15).

The expansion of the dairy industry in Ecuador—resulting from a growing population and its high demand for milk and by-products—meant closer contact among animals. This increased the risk of transmitting *M. bovis* bacilli (23).

Breed-related differences have been reported (1), with BTB infecting a higher proportion of dairy breeds (*Bos taurus*), than indigenous zebu cattle (*Bos indicus*) and crossbred beef cattle. Additionally, the use of the European breeds (e.g., the Holstein Friesian) to improve milk production through artificial insemination, resulted in higher BTB susceptibility (24), indirectly increasing the probability of BTB infections. Beef cattle husbandry systems differ from dairy management and have a decreased risk of infection due to less contact between animals (25). Meat production farms in Ecuador often have expansive pastures and most are located in tropical areas. Table 2 summarizes all available studies conducted on beef cattle in Ecuador (26–36).

All surveys showed a low number of skin reactors, except for two studies in the province of Guayas that showed prevalences of 3.4% and 5.6%. This high percentage can be attributed to the fact that in both studies only SITT was applied and positive reactors were not confirmed by CITT. Some of the reactions, therefore, could have been caused by contact with environmental NTM, especially in young cattle (13, 37).

### **Veterinary inspection**

In geographic areas with high BTB, its prevalence can be estimated by the proportion of macroscopic tuberculous lesions detected during post-mortem examination (followed by the rejection of carcasses and viscera from these animals), if a reliable system exists (9). Indeed, programs based on slaughterhouse surveillance are only effective if they use a reliable traceability system for tracing-back to the herd of origin. The distribution and development of lesions depend on the route of transmission (38) and can vary, although most often located in thoracic lymph nodes due to the infection by respiratory route (39). Therefore, exhaustive veterinary control needs to be established to identify affected animals, i.e., palpation and inspection of lungs, liver, spleen, kidney, mammary gland, and associated lymph nodes (39). Vigilance is recommended, however, since not all infected cattle exhibit gross lesions. On the other hand, detailed meat inspection allows the identification of lesions

## Study 6

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in apparently healthy animals, which increases the number of detected animals and avoids the consumption of BTB-infected cattle (40).

Although veterinary inspection at slaughterhouses potentially constitutes a good method for identifying the presence of TB, it is not routinely implemented in Ecuador and only a few studies on the topic have been published. Those 14 studies are shown in Table 3 (40–52). The studies that took place in 1972 – 2003 were conducted in the provinces located in the tropical areas where meat production is the priority; later studies were performed mainly on dairy cattle in the highlands. The first study, conducted in 1972, entailed veterinary inspection at the slaughterhouse of Guayaquil to identify pigs with BTB (41). A low prevalence of 0.2% was found. More recently, a high proportion of affected dairy cattle were observed in 2007 (2.3%) and 2008 (2.2%) in the highlands of Ecuador, specifically the Mejia canton of the Pichincha province (40). This study applied various laboratory-based diagnostic methods, in addition to the macroscopic investigation, and the presence of *M. bovis* was confirmed (40).

In the Ecuador's dairy areas, 90% of the cattle in the highlands are Holstein Friesian, whereas in the tropical areas, the main breeds used for milk production are Jersey, Brown Swiss, and crossbreeds. The differences in BTB prevalence among cattle breeds and geographic areas present in Ecuador, were also found in the slaughterhouses of Argentina (9), Brazil (53, 54), and Mexico (55, 56).

### Laboratory diagnosis

Laboratory-based techniques are important for improving diagnosis of mycobacteria and identifying the species. In the slaughterhouse survey in the Mejia canton, all positive cultures obtained from tissue samples of tuberculous-like lesions were identified (40). Apart from *M. bovis*, NTM were also identified, i.e., *M. gordonae*, *M. szulgai*, *M. celatum*, and *M. avium-intracellulare-scrofulaceum*; however their clinical relevance was not further investigated. In addition, polymerase chain reaction (PCR) based on 16S rRNA (ribosomal ribonucleic acid) confirmed the presence of *M. bovis* and NTM in lungs and lymph nodes (14).

DNA-fingerprinting analysis allowed identifying different *M. bovis* strains. All *M. bovis* isolates from the Mejia canton surveys displayed the same spoligotype (SB0980) (Proaño-Pérez et al., submitted for publication. Contact the corresponding author for more information).

The homogeneity of the isolates found in this study could reflect a short timeframe of events. *M. bovis* spoligotypes have been reported to change *in vitro* in approximately 60 years (57), which could suggest that BTB was introduced into this dairy area together with new, European cattle breeds in the mid-Twentieth century.

## ZOONOTIC TB

*M. bovis* infection in humans can occur through inhalation of infectious droplets from a live or slaughtered animal or by consumption of unpasteurized dairy or meat products from infected animals (58, 59). People working in animal husbandry, slaughterhouse workers, veterinarians, and people in close contact with possibly-infected animals are at a higher risk for *M. bovis* infection (60). In Argentina, a 2.4% prevalence of zoonotic TB has been reported, with a high association with type of work performed (9). Lack of hygiene, existence of informal trade, absence of veterinary inspection, ignorance of the disease, and deficient use of gloves have been reported as risk factors among slaughterhouse workers (61).

In Ecuador, human cases of TB detected in public and private hospitals are reported to the Ministry of Public Health, which is responsible for the human TB control program and provides free treatment. In 2009, the Ministry of Public Health reported 3 317 microscopy-positive new cases of pulmonary TB, 584 cases of extra-pulmonary TB, and 613 deaths caused by the disease nationwide (62). According to the World Health Organization (WHO), Ecuador reported 4 703 cases of TB in 2009 (63).

The national reference laboratory, “Leopoldo Izquieta Pérez Institute” (LIP), (Guayaquil, Ecuador) identified only two *M. bovis* isolates based on their growth and/or morphological characteristics (in 1998–2005): one from the lymph node of a girl 1 year of age, and the other from a pulmonary specimen of a boy 3 years of age (64). However, it is important to take into account that *M. bovis* requires special conditions to grow *in vitro*. The

## Study 6

appropriate medium to facilitate growth of the bacilli (65) should contain sodium pyruvate, but no glycerol, e.g., Stonebrink medium. The latter medium is not routinely used in the LIP laboratory. Secondly, a number of human *M. bovis* infections might be misdiagnosed because the procedure required for differentiating between *M. bovis* and the other members of the *M. tuberculosis*-complex is not routinely performed.

In 2007, a study was conducted on 157 farm and slaughterhouse workers from the Mejia canton to evaluate TB prevalence by tuberculin skin testing (TST). A positive reaction was seen in 29%. In addition, the risk factors associated with disease transmission among this population were studied. A significant association was documented between TST positivity and masculine gender ( $P = 0.02$ ; odds ratio [OR] = 2.5) and consumption of raw milk ( $P < 0.00$ ) or cheese made by hand/homemade ( $P = 0.003$ ) (66). These findings clearly show that cultural habits play a major role in risk behavior; consumption of raw milk is considered healthier and is widespread among this population group.

## ECONOMIC LOSSES FROM BTB

The national economic losses due to BTB cannot be accurately calculated because standardized data is lacking, and the data that does exist is simply an estimate of BTB prevalence. However, the economic losses in the Mejia canton are estimated at approximately US\$ 460 000 per year. This estimation is based on the true prevalence determined in 2008 by CITT (15) and official data on the cattle population for this canton: approximately 55 000 head of cattle distributed among 2 722 herds (12). Three important factors were considered in estimating economic losses caused by affected cattle: decreased milk production (13%), weight loss (36%), and decreased reproductive rate (12%) (10). Nevertheless, the condemnation of carcasses was not taken into account for this calculation because the BTB-suspected cattle were not always entirely destroyed due to a lack of sanitary controls in some slaughterhouses.

## BTB CONTROL PROGRAM

Today, the quality of dairy products is a high priority for the global dairy industry, as it is for Ecuador's. In Ecuador, the Ministry of Agriculture and Livestock food safety

## Study 6

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department focuses on the quality of animal products. The private dairy sector pays more to dairy farmers who have earned a health certificate, awarded by the Ministry of Agriculture and Livestock, declaring their herd to be free of brucellosis and BTB. In theory, this status should be granted only after two consecutive negative results, meaning disease is not found among the herd, at two 6-monthly controls. In addition, the certification should be granted only by the Ministry of Agriculture and Livestock. However, this is not always the case in practice. Private laboratories offer tuberculin testing for cattle, but the tests are generally not administered by qualified veterinarians. Plus, the quality of PPD is not tested, as there is no reference laboratory for animal diseases to carry out quality control.

In general, BTB control programs are based on the early diagnosis and rapid elimination of positive CITT reactors in farms and at trade. Complementary strategies are recommended, i.e., epidemiological surveillance, proper veterinary supervision at slaughterhouses and cattle markets, and adequate control of cattle movement (67). In Spain, such a policy has been implemented since 1965, resulting in a reduction of BTB prevalence among cattle (68).

In Ecuador, there is no national BTB control program implemented yet. A national policy should be established with an obligatory CITT and culling of positive reactors. However, the implementation of such a national program has economic and logistic limitations. The total cost of a control program cannot be financed by the government alone; it should be shared with the private sector. Financial support should cover all related expenses, i.e., education and public awareness, incentives for farmers to improve the health status of their herds (through the price of the milk), regular CITT, compensation for culling, surveillance monitoring, and research.

## NEXT STEPS

In conclusion, the data presented by this article confirm the presence of BTB in beef and dairy cattle in Ecuador, and justify the implementation of a national health policy to control the disease. A national control program is urgently needed to avoid the spread of the BTB, and is an important step toward promoting international trade of animals and their products. Moreover, these measures would reduce the zoonotic impact of the disease among

## Study 6

the population, especially in high-risk groups living in BTB-prevalent areas of the country. The situation in Ecuador may be similar to that of other countries in the Region of the Americas; thus the information present here could be used in the planning of control and eradication programs elsewhere.

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## Study 6

**TABLE 1. Apparent prevalence of bovine tuberculosis in dairy cattle according to available studies, Ecuador, 1977 – 2008**

Reference	Year	Location		Test used	Herd size <sup>a</sup>	No. of Herds	Positive cattle / No. of animals	(%)
		Province	Canton					
Acosta & Parreño (16)	1977	Tungurahua	Píllaro	SITT <sup>b</sup> & CITT <sup>c</sup>	NA <sup>d</sup>	20	7 / 2 132	0.33
		Pichincha	Cayambe		large	2	5 / 178	2.81
Andino-Ashqui (18)	2001	Imbabura	Otavalo	SITT & CITT	large	1	24 / 329	7.29
		Carchi	Espejo		large	2	11 / 516	2.13
Salazar & Cevallos (20)	2002	Pichincha	Cayambe	SITT & CITT	NA	26	14 / 3 006	0.47
Cano & Chulde (21)	2002	Pichincha	Mejía	SITT & CITT	large	13	152 / 3 089	4.92
			Espejo, Tulcán					
Burbano & León (19)	2002	Carchi	Montufar, Huaca	SITT & CITT	NA	NA	52 / 3 011	1.73
Bedón & Verdesoto (45)	2003	Imbabura	Ibarra, Otavalo	SITT & CITT	large	13	73 / 3 005	2.43
Alemán, et al. (17)	2003	Tungurahua	Píllaro, Mocha	SITT & CITT	NA	24	49 / 4 012	1.22
Proaño-Pérez, et al. (14)	2006	Pichincha	Mejía	SITT & CITT	large	15	26 / 327	7.95
Proaño-Pérez, et al. (15)	2007	Pichincha	Mejía	CITT	large	13	142 / 1 644	8.63
Proaño-Pérez, et al. (15)	2008	Pichincha	Mejía	CITT	large	13	122 / 1 446	8.43

<sup>a</sup> > 70 bovines<sup>b</sup> Simple intradermal tuberculin test<sup>c</sup> Comparative intradermal tuberculin test<sup>d</sup> Not available.

## Study 6

**TABLE 2. Apparent prevalence of bovine tuberculosis in meat production cattle according to available studies, Ecuador, 1977 – 2004**

Reference	Year	Location		Test used	Herd size <sup>a</sup>	No. of Herds	Positive cattle / No. of animals (%)	(%)
		Province	Canton					
Cañizares (26)	1977	Guayas	Guayaquil	SITT <sup>b</sup>	NA <sup>c</sup>	NA	33 / 975	3.38
Maretti (27)	1981	Galapagos	San Cristobal	SITT	NA	NA	0 / 1 000	0.00
Lojan (28)	1982	El Oro	Santa Rosa	SITT	NA	NA	4 / 1 465	0.27
Aguirre (29)	1984	El Oro	Pasaje	SITT	NA	NA	0 / 500	0.00
Torres, et al. (30)	1996	Santo Domingo	Santo Domingo	SITT	large	1	21 / 4 888	0.43
Gutierrez (31)	1997	Guayas	El Triunfo	SITT	NA	NA	0 / 300	0.00
Muñoz (32)	1998	Los Rios	Ventanas	SITT	NA	NA	0 / 320	0.00
Villamar (33)	2000	Manabí	Paján	SITT	NA	NA	0 / 300	0.00
Moncada (34)	2003	Guayas	Naranjal	SITT	NA	NA	14 / 250	5.60
Avellan (35)	2003	Guayas	Bucay	SITT	NA	NA	2 / 200	1.00
Arevalo & Zamora (36)	2004	Santo Domingo	Santo Domingo	SITT & CITT <sup>d</sup>	large	37	20 / 4 029	0.50

<sup>a</sup> > 70 bovines<sup>b</sup> Simple intradermal tuberculin test<sup>c</sup> Not available.<sup>d</sup> Comparative intradermal tuberculin test

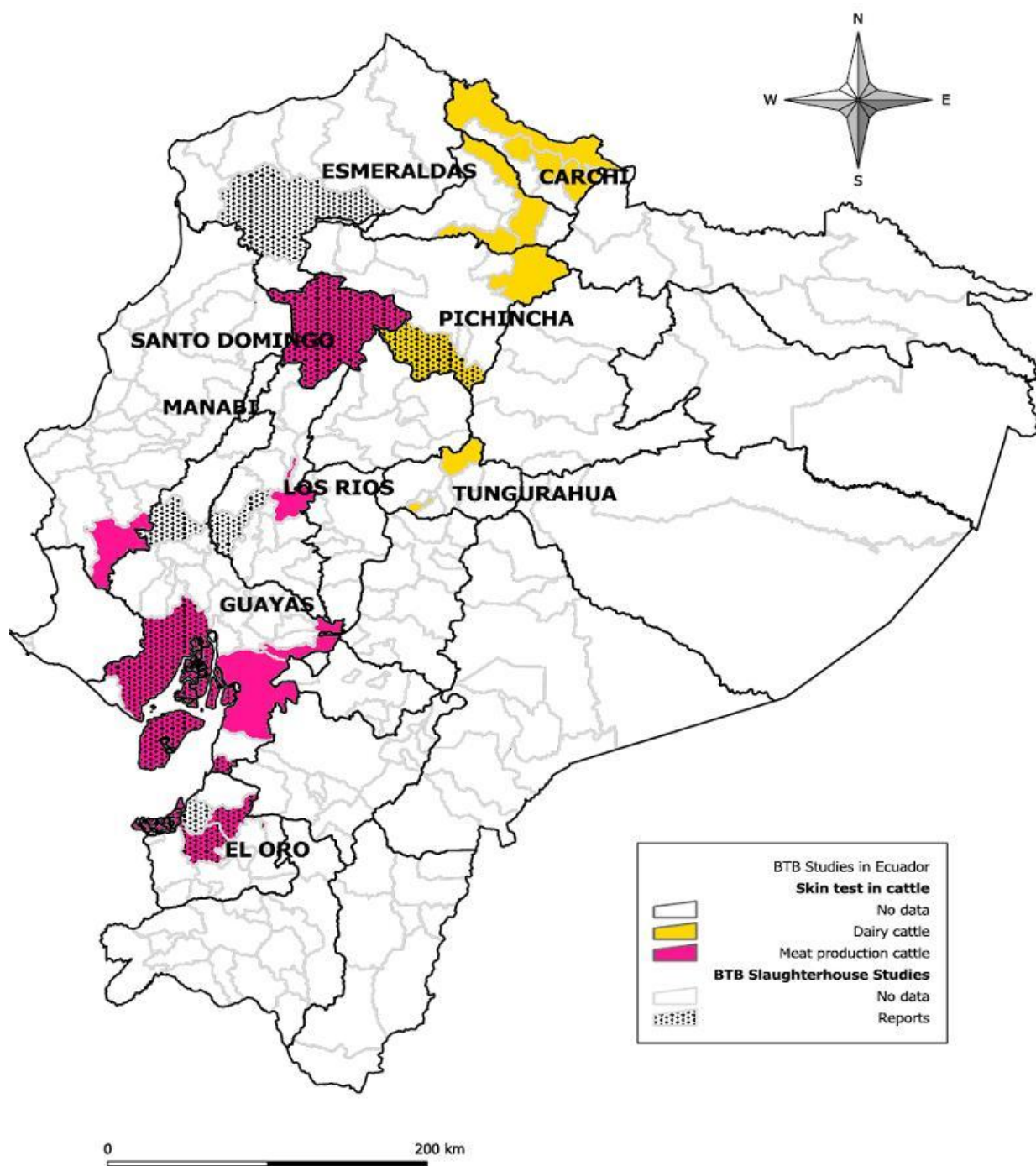
## Study 6

**TABLE 3. Veterinary inspection performed on dairy and meat production animals to identify bovine tuberculosis in slaughtered animals, Ecuador, 1972 - 2008**

Reference	Year	Location		Specie slaughtered	Positive cattle / No. of animals	%
		Province	Canton			
Villacis (41)	1972	Guayas	Guayaquil	porcine	19 / 10 739	0.18
Mata (42)	1973	Guayas	Guayaquil	bovine	16 / 3 425	0.47
Cruz (43)	1985	El Oro	Machala	porcine	0 / 1 500	0.00
Haz (59)	1987	Los Rios	Vinces	porcine	1 / 6 420	0.02
Cueto & Suárez (45)	1993	Guayas	Guayaquil	bovine	28 / 23 029	0.12
Andrade (46)	2000	Guayas	Guayaquil	goats	2 / 550	0.36
Coloma (47)	2000	Santo Domingo	Santo Domingo	bovine	6 / 2778	0.21
Villón (48)	2002	Guayas	Guayaquil	bovine	80 / 4200	1.90
Torres (49)	2002	El Oro	Pasaje	bovine	0 / 1395	0.00
Jaramillo (50)	2002	El Oro	Santa Rosa	bovine	0 / 1047	0.00
Jauregui (51)	2003	Guayas	Colimes	bovine	0 / 283	0.00
Mera (52)	2003	Esmeraldas	Quinindé	bovine	0 / 400	0.00
Proaño-Perez, et al. (40)	2007	Pichincha	Mejía	bovine	16 / 687	2.33
Proaño-Perez, et al. (40)	2008	Pichincha	Mejía	bovine	17 / 703	2.24



**FIGURE 1. Studies conducted using tuberculin skin tests and veterinary inspections to determine prevalence of bovine tuberculosis, Ecuador, 1972 - 2008**



## **CHAPTER 4 – DISCUSSION AND PERSPECTIVES**

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In Ecuador, as in other Latin American countries, BTB is a major concern in the dairy cattle industry, with economic and public health importance. The increase of peri-urban dairy production associated with the absence of active BTB control program in Ecuador are major risk factors in the context of BTB. Moreover, official information related to the epidemiologic situation of BTB in Ecuador is lacking and there is no national veterinary reference laboratory. The present work was undertaken in this context. The objectives were to provide data on the epidemiologic situation of BTB among dairy cattle in northern Ecuador and start up the first national diagnostic laboratory for *M. bovis*. The research was focused on the prevalence and incidence of BTB among dairy cattle herds in the Mejia canton, including an analysis of risk factors, post-mortem examination, laboratory-based diagnostic, molecular epidemiology, and zoonotic implications.

Official data related to BTB in Ecuador are scarce. Currently, there is no national BTB control program based on test and slaughter, notification and systematic surveillance in slaughterhouses. According to de Kantor & Ritacco (2006), Ecuador is in the group of Latin American countries with a supposed relatively high prevalence.

So, in the first step, it was important to determine the prevalence of BTB in northern Ecuador. We conducted a first survey in 1012 cattle from 59 dairy herds (small, medium and large farms) from the Mejia canton, which is one of the most important dairy regions in the country. The single intradermal tuberculin test (SITT) and the comparative intradermal tuberculin test (CITT) were applied to identify reactor cattle. Results demonstrated 7.95 % of tuberculin skin-reactors in large herds *versus* 4.24% in medium and 0.30% in small herds. This higher prevalence of BTB in large herds can be attributed mainly to the closer contact between animals in these farms. In large dairy herds, cattle-to-cattle transmission can be facilitated by

poor ventilation systems in barns and sheds (Goodchild *et al.*, 2001), and also by sharing small sized feeders. Additionally, cattle with stressful conditions related to increased density in small corrals can be more susceptible to BTB infection with *M. bovis* (Parra *et al.*, 2005). These conditions were frequently observed in the Ecuadorian large herds.

During the first study as a skin test survey performed in the Mejia canton, *M. bovis* was isolated for the first time from cattle in Ecuador. Although some skin-testing studies were performed since 1977 on dairy and meat production cattle (Acosta & Parreño, 1977; Cañizares, 1977), *M. bovis* was never isolated nor identified. This can be attributed to the fact that this bacillus requires selective media and needs at least 6 to 8 weeks to grow *in vitro*.

With the background obtained from the first study, we planned a second survey in the same canton including 2022 cattle from 20 medium and large herds. The aim was to determine the real prevalence and the annual incidence of BTB by using CITT during a two years study. The results showed that the true annual incidence was 1.70%, and the true prevalence 7.41 % and 7.13% in 2007 and 2008, respectively; the apparent prevalence in large herds was 8.43% for the year 2008, suggesting an increase of the disease (i.e. the apparent prevalence in large herds was 7.95% in 2006, and 8.43% in 2008). These data underline the importance to apply test-and-slaughter schemes to avoid the spread of BTB in the area (Collins, 2006), and to evaluate herd cattle rather than individual animals.

The threshold for a positive tuberculin skin test was the internationally accepted one of  $\geq 4$ mm, however it has been argued that optimal thresholds may vary by setting (i.e., by breed and specific PPD) (Streiner & Cairney, 2007). In Ethiopia, the true prevalence observed at a threshold of  $>2$ mm was 19.6% compared to 18.5% when using a threshold of  $>4$ mm,

resulting in increased sensitivity without a loss of specificity according to this study (Ameni *et al.*, 2008). We therefore next set out to evaluate the specific cut-offs according to the conditions in Ecuador and taking into account factors that could interfere in the reactions such as technique, management and origin of PPDs, and presence of NTM.

In the second study, possible risk factors for BTB were also investigated. The CITT results were compared with individual (i.e. lactation, age) and herd-level (i.e. herd size, new cattle introduction and contact with other species of animals) risk factors through a multivariate logistic regression. Herd size was identified as a significant BTB risk factor in dairy cattle, confirming the results of the first study. Large herds with more than 70 cattle had a higher prevalence of infection than medium herds ( $P < 0.01$ ). The number of reactors also increased significantly with age ( $P = 0.03$ ). In addition, lactating cows presented a higher risk to develop a positive PPD reaction in large herds than in medium herds (OR = 1.75). This result highlights the need to improve the management in large herds especially during the lactation period. According to Kazwala *et al.*, (2001), stressful conditions associated with high milk production have to be considered. Other risk factors such as contact with other animal species (carnivores, small ruminants or pigs) were also identified. It is very common to find different species of domestic animals living in close contact in the farming systems in Ecuador. Contact with wildlife is an important risk factor for BTB recognized in many studies, however in our survey information obtained from farmers was inadequate to assess this risk factor; this could be addressed in a future study.

While the results of these studies show the scope of BTB in Ecuador and suggest husbandry strategies that could diminish it, the risks from BTB are not recognized by cattle owners, farm

and slaughterhouse workers. Information campaigns in rural areas will be key components in the overall strategy for the control of BTB in Ecuador.

In the third paper, we evaluated the different routes of transmission of *M. bovis* in cattle, drawing inferences from the patterns of lesions in slaughtered animals (Neill *et al.*, 1994). Moreover, we sampled lesions and compared available BTB diagnostic tools.

Veterinary inspection was carried out in the local abattoir of the Mejia canton. The detection of macroscopic lesions should normally be performed by the veterinarian inspectors. However in Ecuador, this procedure is not systematically followed because the veterinarians are not trained in it and also because it requires additional time in the process of slaughter. In our survey, detailed veterinary inspection was performed and all suggestive lesions were sampled. Visible lesions were mainly observed in thoracic lymph nodes suggesting that the respiratory tract is the main route of transmission in dairy cattle from this area. In developed countries, *M. bovis* infection in cattle is mostly confined to the respiratory system, where a single bacillus transported within a droplet is probably sufficient to establish infection in the lung (Menzies *et al.*, 2000). The transmission by respiratory route could be reduced by appropriate management, proper ventilation in closed areas, and reduction of close contact between animals (Phillips *et al.*, 2003; Goodchild *et al.*, 2001).

Although slaughterhouse inspection is essential in BTB control programs, visible lesions are not present in all infected animals and infections with NTM can be mistaken for BTB lesions (Corner, 1994; Whipple *et al.*, 1996; Asseged *et al.*, 2004; Teklul *et al.*, 2004). Therefore, *in vivo* tests in farms (i.e., CITT) associated with post-mortem inspection at the slaughterhouse could improve the detection rate of infected cattle. Only an official control program could

promote this practice as a routine procedure during slaughtering, and by this way detect and reject carcasses from affected animals. In a program based on slaughterhouse inspection, trace back of tuberculous animals to herds of origin is crucial. Its success is highly dependent on an effective animal identification system such as individual ear tags. This system is not yet implemented in Ecuador.

To evaluate the diagnostic laboratory tools currently available to identify *M. bovis*, we analyzed the samples collected at the abattoir during veterinary inspection. The tissue samples were examined for the presence of mycobacteria by microscopy, histopathology, *in vitro* culture and PCR.

Culture remains the gold standard method for confirmation of BTB infection. The diagnostic accuracy of a test is primarily defined by its sensitivity (Se) and specificity (Sp). Compared to culture, the three other diagnostic tools presented a lower Se. Even if culture is the gold standard, the Se of detection by culture can be reduced due to the decontamination procedure, storage conditions, and number of freeze-thaw cycles of the samples, which could affect the viability of the mycobacteria (Portaels *et al.*, 1988). The Se of PCR can also be influenced by e.g. insufficient bacillary load or inhibitors present in the samples (Mangiapan *et al.*, 1996; Cardoso *et al.*, 2009), whereas the Se of microscopy and histopathology can be strongly influenced by the technique used and performance of the technician (Demelash *et al.*, 2009).

A survey kappa analysis of the laboratory tests revealed a high concordance between culture and PCR ( $\kappa = 0.61$ ), a moderate concordance between culture and histopathology ( $\kappa = 0.49$ ), culture and microscopy ( $\kappa = 0.44$ ), PCR and microscopy ( $\kappa = 0.47$ ), and PCR and histopathology ( $\kappa = 0.44$ ). Nevertheless, we observed a low concordance between microscopy

and histopathology ( $\kappa = 0.20$ ). Most positive samples by culture were confirmed by PCR, although not always the same samples were positive in all tests which highlights the importance of applying more than one test to confirm the presence of *M. bovis*. These results showed that more sensitive tests need to be developed for BTB control/eradication programs.

To complete the study, spoligotyping was used to compare the strains of *M. bovis* isolated from the Mejia canton. This technique was performed in the Mycobacteriology Unit of the Institute of Tropical Medicine, Antwerp, Belgium. Surprisingly, all *M. bovis* isolates showed the same spoligotype named SB0980 according to the *M. bovis* molecular typing database ([www.mbovis.org](http://www.mbovis.org)). It lacked spacers 3,9,16 and 39 to 43. The absence of these spacers is characteristic of *M. bovis* isolates from South America and Spain (Zumarraga *et al.*, 1999). High clustering rates, i.e. high numbers of isolates showing identical fingerprints, has been described as an indication of active transmission (van Soolingen *et al.*, 1994). The identical spoligotypes encountered in Latin American and European countries suggest relative recent transmission of these strains. This would coincide with the import of several European cattle breeds in Latin American countries since the 19<sup>th</sup> century. The same strain found in different countries can reflect the active trade of livestock between these regions (Zumarraga *et al.*, 1999). Only systematic typing of all *M. bovis* isolates from the country, would allow for proper identification of the causative agent and demonstration of geographical and chronological heterogeneity of strains playing a role in BTB in Ecuador.

Human public health can also be affected by BTB, especially in rural inhabitants who live in direct contact with infected cattle. The zoonotic impact of BTB on public health was one of the principal reasons for the earlier introduction of direct inspection methods in meat hygiene control (veterinary inspection) and the pasteurization of milk (Collins, 2006). Nevertheless,



although the pasteurization method has reduced the transmission of *M. bovis* from cattle to humans, the increased incidence in cattle makes exposure of human populations more likely (Cornejo *et al.*, 1998). In addition, wildlife may be a source of infection, particularly in countries where bush-meat is eaten.

It is estimated that 24% of the Latin American bovine population resides in areas devoid of proper BTB control programs (Gil & Samartino, 2001). In Argentina, two percent of human TB cases have been recorded as caused by *M. bovis* (de Kantor & Ritacco, 2006). To evaluate the situation in human populations from the Mejia canton, we performed a survey among farmers and abattoir workers. Tuberculin skin testing revealed a high prevalence of reactors (29%), indicating a very high exposure risk in this population. Most of them were living in a farm with at least one bovine CITT reactor. The main risk factors associated with positive human reactors were consumption of raw milk coming directly from the same farm where they work or by cheese manufactured in a traditional manner. However, we did not isolate any *M. bovis* strain in slaughterhouse or farm workers. The presence of *M. bovis* in tissues from infected cattle represents a high risk in abattoir workers as they may be exposed to the bacteria during slaughtering. These animals should be slaughtered following strict safety procedures to avoid human infection. In the meat industry, airborne infection is frequent among slaughterhouse and farm workers (Thoen *et al.*, 2006).

Human TB caused by *M. tuberculosis* or *M. bovis* is clinically, radiologic and pathologically indistinguishable (Wedlock *et al.*, 2002). Human-human infection with *M. bovis* is usually considered as a rare event. However, the high prevalence of HIV combined with the presence of several risk factors could change the epidemiology of the disease, increasing the human-human transmission (Ayele *et al.*, 2004). In Ecuador, the estimation of the number of human

cases due to *M. bovis* is unknown. Only two human *M. bovis* cases were reported by the Nacional Laboratory of Hygiene “Leopoldo Izquieta Pérez”. The finding was a result of a study performed on strains stored in the period from 1998 to 2005, which identified *M. bovis* in two children: one from a lymph node and the other from a pulmonary specimen (de Kantor *et al.*, 2008).

Control programs of human TB (regardless of the causative agent) are primarily based on clinical diagnosis supported by X-Ray analysis and/or laboratory-based methods. It is known that *M. bovis* may be involved more frequently in extra-pulmonary disease. In developing countries however, the frequency and involvement of *M. bovis* in non-pulmonary TB is not quantified because of the limited laboratory facilities for culture and identification of tubercle bacilli (Cosivi *et al.*, 1998). The *in vivo* diagnosis (tuberculin skin test) is an important procedure in the control of the disease that needs to be complemented with available laboratory tools. Treatment of human TB is done following standard short course chemotherapy, which has been attempted with a 95% cure rate for drug-sensitive bacilli. In developing countries, the Bacille-Calmette-Guerin (BCG) vaccine has been used in children to protect against severe disease, especially in areas with a high prevalence of TB. Although it is considered to have moderate efficacy, BCG vaccine is the only licensed vaccine against TB to use in humans.

While treatment has been studied in cattle, it is not considered as rational approach as test and slaughter programs. Vaccination can be used in cattle to reduce the spread of infection in cases where the incidence of TB is high and when eradication program cannot be organised. In this case, vaccination is used as a temporary measure. Although in most countries vaccination is not applied in cattle because lack of efficacy and also because BCG vaccines

compromise the interpretation of the tuberculin skin test (Mongham *et al.*, 1994). However, BCG vaccines can be used in wildlife reservoirs to reduce the infection (Amanfu W., 2006), this procedure is important to apply because *M. bovis* in wildlife may complicate control/eradication programs of BTB in cattle (Thoen, 1997), especially in developed countries.

*M. bovis* is a serious constraint in the international trade of animals and their products, and causes major economic losses to livestock. In Ecuador, dairy cattle management leads to diseases such as BTB due to the stress induced by intensive milk production and the prolonged lifetimes of dairy cows. The main factors considered to raise the prevalence of BTB are the expansion of the dairy industry caused by the high demand of milk and byproducts, the intensification of the farms increasing the cattle population, the presence of *M. bovis* in livestock and the lack of control measures. In Iran, in a relative short time, *M. bovis* has emerged as a major cause of cattle illness and economic important loss, caused by increased host susceptibility, changes in farming practices (i.e., intensification), and frequent escape of *M. bovis* from skin test-slaughter-scheme (Tadayon *et al.*, 2008). As other countries face similar risks, implementation of nation-wide BTB control programs is urgently needed.

The control of BTB has been included in the Millenium Development Goals; this activity will be reached through the Veterinary Public Health team of the Food and Agriculture Organization (FAO). The main actions to take into account to achieve this goal could involve: (1) to increase biosecurity measures on farms to avoid BTB infections and improve the animal health, (2) to implement or improve animal testing by increasing the sensitivity and specificity of diagnostic test available, and (3) to develop a wildlife surveillance network (Humblet *et al.*, 2009). Nowadays, the concept “One World, one Health” is being adopted to control several

zoonoses, which establishes a more interdisciplinary and cross-sectoral approach to prevent epidemic or epizootic diseases. Within this concept, medical doctors and veterinarians can join forces to fight against BTB control and its eradication.

In some Latin American countries control programs are being implemented. In Argentina, the control program is based on a close cooperation among animal health services, farmer organizations, veterinary associations, veterinary schools, and milk processing plants. One serious drawback remains: no official compensation for culled animals. This represents an important problem that could jeopardize the program (de Kantor & Ritacco, 2006). Taking into account these experiences, a proposition for a national control program in Ecuador could be based on the following topics: (1) to train and qualify veterinarians to perform early *in vivo* diagnosis (i.e., CITT) and detailed post-mortem inspection, (2) to create a reference laboratory for BTB for detection and identification of the bacillus and stimulate investigation of the disease, (3) to regularly perform skin testing and culling of reactors in combination with control movement, (4) to improve animal identification and record keeping systems in a centralized database, (5) to establish a trace-back system to identify the source of infection for animals detected at slaughter, (6) to develop an official compensation for culled animals through financial cooperation of the public and private sectors, and (7) to implement an education campaign with emphasis on populations at risk.

Finally, by applying this holistic approach, BTB could be under control in Ecuador; first in prevalent regions and then at a national level. However, without strengthened control measures *M. bovis* is unlikely to disappear in Ecuador.

## **CHAPTER 5 – SYNOPSIS**

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Bovine tuberculosis (BTB) is a chronic infectious disease with worldwide distribution that mainly affects cattle. However, it can also be present in humans, domestic animals and wildlife. BTB is considered as an important disease with socio-economic and public health importance. BTB significantly affects the international trade of animals and their products, and causes major economic losses to those directly or indirectly involved in that trade. In developing countries from Latin America and Africa, BTB is underestimated due to the lack of reporting and active surveillance for the disease. The prevalence and epidemiology of BTB in Ecuador have remained unknown, because the disease has not been declared yet as animal and public health importance. The aims of this thesis were to provide data on the epidemiologic situation of BTB among dairy cattle in northern Ecuador, to perform laboratory-based analyses to identify the bacillus, to evaluate risk factors and provide the basic tools to create a control program for BTB in Ecuador. Our results are presented as five original studies and one review.

**(i) Preliminary observations on *Mycobacterium* spp. in dairy cattle in Ecuador.**

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Freddy Proaño-Pérez, Leen Rigouts, Jef Brandt, Pierre Dorny, Jorge Ron, Maria-Augusta Chavez, Richar Rodriguez, Krista Fissette, Anita Van Aerde, Françoise Portaels and Washington Benitez-Ortiz

This study aimed to evaluate the situation of bovine tuberculosis in Mejia canton, a major dairy cattle production region in Ecuador. Randomly selected cattle (1012 from 59 farms), classified according to herd size, were tested by the single tuberculin test (STT). Sixty days later, positive reactors were tested again by the comparative tuberculin test (CTT). In addition, tissue samples from 2 STT-CTT-positive reactors detected on a farm were necropsied in a local slaughterhouse and analyzed bacteriologically. On average, 4.24% cattle

reacted positive by STT; 3.85% positives were detected by CTT, the highest number (7.95%) in large herds vs. 3.4% in medium and 0.3% in small herds. At necropsy, *Mycobacterium bovis* was isolated in mesenteric lymph nodes and the lungs of one animal. 16S rRNA-gen-based PCR confirmed culture results and allowed to differentiate mycobacteria other than tuberculosis (MOTT). This study confirms the importance of tuberculosis in Ecuadorian dairy cattle with herd-size likely to be a crucial parameter in the prevalence of the disease. The implementation of a national control program is necessary and should be based on the detection of positive cattle by in vivo tests such as STT in combination with CTT, which need to be applied as a routine procedure to detect tuberculous cattle, followed by culling.

**(ii) Comparative intradermal tuberculin test in dairy cattle in the north of Ecuador and risk factors associated with bovine tuberculosis**

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Freddy Proaño-Pérez, Washington Benitez-Ortiz, Maritza Celi-Erazo, Lenin Ron-Garrido, Ricardo Benitez-Capistros, Françoise Portaels, Leen Rigouts and Annick Linden

We investigated the prevalence of bovine tuberculosis (BTB) in Mejia canton, the major dairy cattle production area in northern Ecuador. Twenty dairy herds comprising 2022 cattle were selected. In 2007 each animal was tested using the comparative intradermal tuberculin test (CITT). In 2008 a follow-up test was performed in the same herds. The true annual incidence was 1.70%, and the true prevalence was 7.41% and 7.13% in 2007 and 2008, respectively. The prevalence was 0.27% and 0.57% in medium-sized herds in 2007 and 2008 respectively compared to 8.63% and 8.43% in large herds ( $P < 0.01$ ). The number of skin-test-positive cases also increased significantly with age ( $P = 0.03$ ), contacts with other species of animals ( $P < 0.01$ ) and introduction of new cattle ( $P = 0.04$ ). Herd prevalence was 55% in 2007 and

65% in 2008. The true annual incidence showed that the disease is increasing because control measures are not yet established. The risk factor analysis detected the main reasons involved in the spread of BTB, only an improved livestock husbandry could avoid these factors and reduce the occurrence of *M. bovis* infections. This study demonstrates the poor knowledge of cattle farmers about this zoonosis, and the necessity for a national BTB-control program in Ecuador.

**(iii) Post-mortem examination and laboratory-based analysis for the diagnosis of bovine tuberculosis among dairy cattle in Ecuador.**

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Freddy Proaño-Pérez, Washington Benitez-Ortiz, Daniel Desmecht, Marco Coral, Julio Ortiz, Lenin Ron, Françoise Portaels, Leen Rigouts, Annick Linden

Veterinary inspection in slaughterhouses allows for the detection of macroscopic lesions reminiscent of bovine tuberculosis, but the presence of *Mycobacterium bovis* must be confirmed by laboratory methods. This study aimed at comparing the performances of the standard diagnostic tools used to identify *M. bovis* in tissue specimens sampled from suspicious animals. During a two years period, 1390 cattle were inspected at the Machachi abattoir in the Mejia canton – Ecuador. A total of 33 animals with granulomatous lesions were detected, representing 2.33% (16/687) and 2.42% (17/703) animals examined in 2007 and 2008, respectively. Ninety-four tissue specimens were sampled and screened for the presence of mycobacteria. Acid-fast bacilli were identified in one third of the suspicious cattle (11/33) and suggestive microscopic lesions in 27.3% (9/33) of the samples examined by direct microscopy and histopathology, respectively. Culturing on Stonebrink medium and 16S-rRNA-based polymerase chain reaction (PCR) yielded 36.4% (12/33) and 27.3% (9/33) of



positives, respectively. Compared to culture, other diagnostic procedures displayed a lower sensitivity, with 56.5% for PCR, and 43.5% for direct microscopy and histopathology; however, the specificity was higher (94.4% for PCR and microscopy, and 97.2% for histopathology). We conclude that reliable post-mortem laboratory testing either requires the combination of a set of available diagnostic tools or necessitates the development of improved new-generation tools with better sensitivity and specificity characteristics.

**(iv) Single *Mycobacterium bovis* strain prevailing among dairy cattle in northern Ecuador.**

*Journal of Preventive and Veterinary Medicine*, 2011, submitted

Freddy Proaño-Pérez, Washington Benitez-Ortiz, Françoise Portaels, Leen Rigouts, Annick Linden

Spoligotyping has been used as an important tool for molecular epidemiology studies on BTB in several countries. In our study, a single *M. bovis* strain prevailing among cattle was demonstrated (spoligotype SB0980). This low polymorphism could be related to the relatively small study area, a common source of infection for the studied cattle population, the fact that no new live cattle was imported in this region and/or the absence of a wildlife source infecting cattle. Therefore, it is necessary to perform molecular epidemiology studies in the rest of the country to identify if more spoligotypes are present and establish geographical distribution and chronological heterogeneity.

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**(v) Risk analysis for infection of *Mycobacterium* spp. in a human population in Ecuador.**

In preparation

Benítez-Capistros Ricardo, Proaño-Pérez Freddy, Celi-Erazo Maritza, Anchatipán-Tapia Fabricio, Ron-Garrido Lenin, Linden Annick, and Benítez-Ortíz Washington

Tuberculosis (TB) in humans is mainly caused by *Mycobacterium tuberculosis*. However, the pathogenic agent of bovine TB, *Mycobacterium bovis*, can also cause the disease in human beings (zoonotic TB). This study was carried out in 157 people, conformed by farm workers and slaughterhouse workers from Mejia canton, considered an important dairy area in Ecuador located in Pichincha Province. The purpose of this research was to evaluate the prevalence of *Mycobacterium* spp. in risk populations by tuberculin skin test (TST) and to analyze the risk factors associated with the transmission of the disease. The results establish a significant association among the positivity of TST to masculine gender ( $P=0,026$ ), consumption of milk coming from farms ( $P=0,030$ ), consuming raw milk ( $P=0,000$ ), and consumption of elaborated cheeses made by hand ( $P=0,003$ ). Consumption of raw milk gave an *odds ratio* of 4,65 [C.I.= 2,01-10,75 at 0,95]. The prevalence to *Mycobacterium* spp. was estimated in 29% [C.I.= 22-36% at 0,95] for this population. In this study the tuberculin skin test results had a high correlation with consumption of raw milk; however, we did not isolate any *M. bovis* strain in slaughterhouse or farm workers. Human specimens require culture and identification of the isolated mycobacterial species in order to apply the accurate treatment. Although introduction of milk pasteurization and meat hygiene control has reduced cases of BTB from cattle to human beings, in rural areas from Ecuador these procedures are not always implemented in rural areas from Ecuador. The estimation of the number of human cases due to *M. bovis* is unknown in the country and further studies are needed to evaluate the real situation.

**(vi) Review article: Situation of bovine tuberculosis in Ecuador**

*Pan American Journal of Public Health*, 2011, 30(3):000-00.

Freddy Proaño-Pérez, Washington Benítez-Ortiz, Françoise Portaels, Leen Rigouts, Annick Linden

Bovine tuberculosis (BTB) is a chronic and contagious disease caused by *Mycobacterium bovis* that affects domestic animals, wildlife and humans. *M. bovis* is a serious constraint in the international trade of animal products, and causes major economic losses to those making their living from livestock. Moreover, in developing countries where BTB controls are lacking, *M. bovis* is also of public health concern. In most developing countries, the prevalence of BTB in livestock is unknown because the information is either not reported or not available. In Ecuador, there is neither national BTB control nor elimination programs. This article reviews the situation of BTB in different regions of Ecuador, by presenting exhaustive data from both tuberculin testing surveys and slaughterhouses surveillance studies since 1972 till 2008. The expansion of peri-urban dairy industry caused by the high demand of milk and byproducts, the intensification of the farms increasing the cattle population, the presence of *M. bovis* and the lack of control caused a raise in the prevalence of BTB in northern Ecuador, and calls for the implementation of a nation-wide BTB control program. The program planned should combine *in vivo* skin testing before slaughter to identify early infections with *M. bovis*, with extended post-mortem inspection including laboratory-based diagnosis. It will only be possible to avoid the spread of the disease and to be able to export and trade products from animal origin if control measures proposed in this thesis are implemented.

## **CHAPTER 6 – REFERENCES**

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